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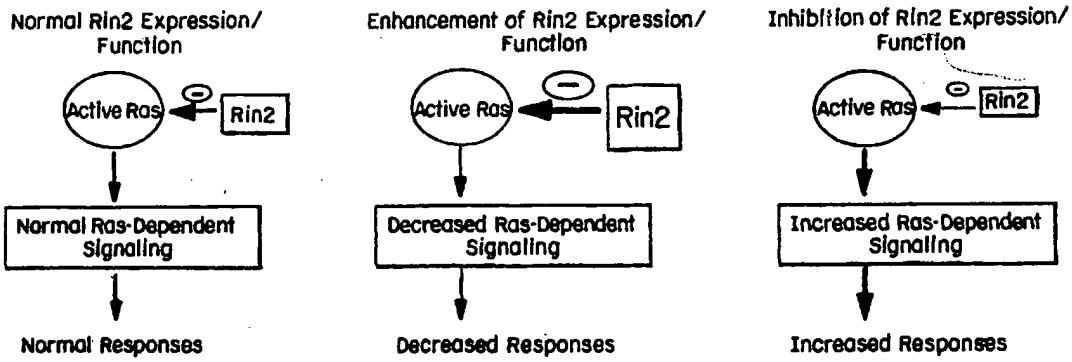
(54) Title: *RIN2*, A NOVEL INHIBITOR OF RAS-MEDIATED SIGNALING



Examples of normal responses regulated by Ras-dependent signaling	Settings in which it is desirable to decrease such responses	Settings in which it is desirable to increase such responses
Mast cell mediator production	Asthma, hay fever, atopic dermatitis	Immunity to pathogens
T cell function	Graft rejection; T cell dependent diseases (certain autoimmune diseases, contact dermatitis, graft-versus-host disease)	HIV infection; certain other infections; immunosupressions associated with cancer therapies
Cell proliferation	Cancer; proliferation of vascular wall cells in arteriosclerosis	Wound healing; angiogenesis (e.g. to improve ischemia; re-epithelialization); nerve regeneration

(57) Abstract

Novel gene, *Rin2*, and encoded protein are disclosed which can inhibit the functional response induced by Ras-dependent signaling pathways are disclosed. Methods of inhibiting or enhancing Ras-dependent signaling and methods of treatment utilizing *Rin2* are also

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RIN2, A NOVEL INHIBITOR OF RAS-MEDIATED SIGNALING

RELATED APPLICATIONS

The present application is a Continuation-in-Part of U.S. Application Serial No. 08/942,819, filed October 2, 1997. The present application also claims the benefit of U.S. Provisional Application No. 60/058,520, filed September 11, 1997. The teachings of the prior applications are incorporated herein in their entirety.

GOVERNMENT FUNDING

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BACKGROUND OF THE INVENTION

The Ras family of small guanine-nucleotide binding proteins plays a pivotal role in many intracellular signal transduction pathways, including those which regulate cellular growth and differentiation and those which contribute to cell activation (Bourne *et al.*, *Nature 348*:125-132 (1990); Marshall, *FASEB J.* *9*:1311-1318 (1995)). Moreover, many different receptors expressed on the surface of diverse cell types can result in the activation of signal transduction pathways that are importantly influenced by Ras, and these pathways in turn determine whether, and to what extent,

these cells respond to such cell surface receptor-dependent activation by proliferating, differentiating (i.e., developing new functional characteristics), and/or expressing specific functions (Figure 1).

Depending on the circumstances, these "down-stream" consequences of the activation of Ras-dependent signal transduction pathways can have either adaptive (physiological) or maladaptive (pathological) consequences. For example, controlled Ras-dependent cellular proliferation is required for wound healing, whereas poorly regulated Ras-dependent cellular proliferation can result in the development of cancer and other neoplasms. Similarly, appropriate Ras-dependent cell secretion of histamine, serotonin, cytokines and other mediators can be important for host defense against parasites and other pathogens, whereas the inappropriate activation of these same pathways, for example, by a reaction to a bee-sting in patients who are allergic to components of bee venom, can lead to fatal anaphylaxis. Thus, Ras represents a major regulator of many of the most fundamental biological processes involved in both health and disease.

The mechanism by which Ras regulates such processes, through interactions with other intracellular molecules, is quite complex. Ras proteins are membrane-associated proteins that cycle between an active GTP-bound form and an inactive GDP-bound form. As illustrated in Figure 1, evidence is accumulating for the existence of many different classes of positive or negative regulators of Ras and positive or negative Ras signaling effectors, all of which, by definition, are thought to interact directly with the active GTP-bound

form of Ras to influence cellular signaling for growth, differentiation and expression of function (Boguski and McCormick, *Nature 366*:643-654 (1993); Marshall, *FASEB J. 9*:1311-1318 (1995); Marshall, *Curr. Opin. Cell Biol 8*:197-204 (1996)).

For example, some of the best-characterized Ras regulators include the GTPase activating proteins (GAPs) and guanine nucleotide exchange factors (Boguski and McCormick, *Nature 366*:643-654 (1993)). The GAPs represent a family of Ras-binding proteins which stimulate the intrinsic rate of Ras GTP hydrolysis and thus negatively regulate the Ras-induced signaling by accelerating the conversion of active GTP-bound form of Ras to the inactive GDP-bound form. Recent studies have identified several GAPs specific for Ras proteins, which include p120-Ras GAP, neurofibromin (the protein encoded by the neurofibromatosis type 1 (NF1) gene), Gap1, Ral-GDS, Rsbs 1, 2, and 4, Rin1, MEKK-1, and phosphatidylinositol-3-OH kinase (PI3K) (Boguski and McCormick, *Nature 366*:643-654 (1993)).

In contrast to Ras regulators, which function primarily by influencing the amount of Ras which is in the GTP-bound active, as opposed to the GDP-bound inactive, form, Ras effectors are thought to influence the ability of active, GTP-bound Ras to initiate signaling (Figure 1). In the case of many Ras-interacting proteins which can influence the intensity of Ras-dependent signaling, it is not yet clear to what extent they function as effectors as opposed to regulators; such proteins can therefore be called Ras regulators/effectors (Boguski and McCormick, *Nature 366*:643-54 (1993); Han and Colicelli, *Mol. Cell. Biol.*

15:1318-1323 (1995); Marshall, *FASEB J.* *9*:1311-1318 (1995); Marshall, *Curr. Opin. Cell. Biol.* *8*:197-204 (1996)).

Ras is important in critical cell signaling events in many cell types. For example, mast cells are important effector cells in IgE-dependent immune responses and allergic diseases (Galli, *New Engl. J. Med.* *328*:257-265 (1993)), and mast cells also contribute to host defense against parasites and bacteria (Echtenacher *et al.*, *Nature* *381*:75-77 (1996); Malaviya *et al.*, *Nature* *381*:77-80 (1996); Galli and Wershil, *Nature* *381*:21-22 (1996)). Mast cells reside in virtually all vascularized tissues and express on their surface the high affinity receptor for IgE (FceRI). Aggregation of FceRI in mast cells by the interaction of receptor-bound IgE with specific multivalent antigen triggers the functional activation of mast cells, which results in the release of a spectrum of biologically active mediators (Ravetch and Kinet, *Ann. Rev. Immunol.* *9*:457-492 (1991); Galli, *New Engl. J. Med.* *328*:257-265 (1993); Beaven and Metzger, *Immunol. Today* *14*:222-226 (1993)). Thus, mast cells activated by FceRI-dependent mechanisms undergo degranulation, resulting in the release of preformed mediators, such as serotonin (5-HT) and/or histamine, the metabolism of arachidonic acid, leading to the release of newly synthesized lipid mediators, and the transcription, translation and secretion of several cytokines (Gordon *et al.*, *Immunol. Today* *11*:458-464 (1990); Galli, *New Engl. J. Med.* *328*:257-265 (1993); Paul *et al.*, *Adv. Immunol.* *53*:1-29 (1993)).

Knowledge of the signaling pathways which result in the FcεRI-dependent secretion of mast cell mediators is increasing. In mast cells and basophils, a type of circulating leukocyte which shares many biochemical and functional characteristics with mast cells (Galli, *New Engl. J. Med. 328*:257-265 (1993)), the FcεRI receptor is a tetrameric complex comprised of a single 45 kDa α chain, which binds the Fc portion of IgE, a single 30 kDa β chain, and a homodimer of two 10 kDa γ chains (Ravetch and Kinet, *Ann. Rev. Immunol. 9*:457-492 (1991); Beaven and Metzger, *Immunol. Today 14*:222-226 (1993)). The β and γ chains contain immunoreceptor tyrosine-based activation motifs (ITAM) which couple the receptor to the src family of protein tyrosine kinases (PTK) p56lyn and p72syk (Beaven and Baumgartner, *Curr. Opin. Immunol. 8*:766-772 (1996)). The FcεRI-dependent activation of these PTKs in turn activates various downstream effector pathways, including those involving PLCγ1 and the MAP kinase pathway (Beaven and Baumgartner, *Curr. Opin. Immunol. 8*:766-772 (1996)).

Recent studies have shown that the crosslinking of FcεRI in mast cells by IgE and specific antigen also results in the activation of Ras and of the associated Shc-Grb2-Sos pathway, which precedes Ras activation, and that the activation of this pathway is dependent on Syk (Jabril-Cuenod *et al.*, *J. Biol. Chem. 271*:16268-16272 (1996)). These results suggest that in FcεRI-activated mast cells, as in T cells and B cells (the major types of lymphocytes responsible for cellular and humoral immunity) which have been activated via the T cell receptor or B cell receptor, respectively, the Shc-Grb2-

Sos pathway can activate the MAP kinase pathway via the activation of Ras.

An effector pathway of Ras mediated by the Raf-1/Erk-activating kinases (MEKs)/Erk-MAP kinases cascade has been well-characterized in numerous systems (Treisman, *Curr. Opin. Cell Biol.* *8*:205-215 (1996)), and this Ras-mediated pathway is important in the mast cell activation and mediator secretion that is induced by IgE- and antigen-dependent aggregation of FcεRI in these cells (Tsai et al., *Eur. J. Immunol.* *23*:3286-3291 (1993); Offermanns *et al.*, *J. Immunol.* *152*:250-261 (1994); Hirasawa *et al.*, *J. Immunol.* *154*:5391-5402 (1995)). In addition, recent studies with rat RBL2H3 mast cells have shown that the FcεRI induction of Ras activation leads to transcriptional activation mediated by the transcription factors Elk-1 and the nuclear factor of activated T cells (NFAT) (Turner and Cantrell, *J. Exp. Med.* *185*:43-53 (1997)). These Ras-dependent signaling pathways in mast cells appear to be complex. Thus, activation of the Raf-1/MEK/Erk cascade appears to be necessary and sufficient for the activation of Elk-1 activity in mast cells. However, the induction of NFAT by FcεRI in mast cells is also mediated in part by Rac-1, which is a putative Ras effector and a member of the Rho family of GTP binding proteins (Turner and Cantrell, *J. Exp. Med.* *185*:43-53 (1997)). Thus, the specific signaling pathways involved in FcεRI-mediated mast cell activation are still unclear.

SUMMARY OF THE INVENTION

As described herein, mRNA differential display was used to identify the cDNA for a novel gene, *rin2* (also

called Rabex-5). As also described herein, expression of *rin2* was shown to be rapidly increased in mouse mast cells activated through FcεRI. Transfection of mouse mast cells with an antisense *rin2* expression vector, which resulted in antisense inhibition of Rin2 expression, potentiated the Ras-mediated intracellular signaling responses that were induced by FcεRI aggregation, such as the induction of c-*fos* expression and the activation of Erk-MAP kinase, JNK and p38 MAP kinase activity. In addition, antisense inhibition of *rin2* expression in mouse mast cells significantly enhanced the amounts of preformed mediator (serotonin) and cytokine (IL-6) released from these cells upon FcεRI-dependent stimulation, suggesting that Rin2 exerts its effects by down-regulating the functional responses elicited by FcεRI aggregation in mast cells. These results support the role of Rin2 as a novel negative regulator/effector of Ras in mast cells and indicate that Rin2 interacts directly the mammalian H-Ras protein.

Moreover, as also described herein, expression of *rin2* is also increased in mast cells stimulated via activation of their major growth factor receptor (i.e., c-kit), in PC12 adrenal pheochromocytoma cells activated via the receptor for nerve growth factor (NGF) (i.e., TrkA), and in T cells activated via the T cell receptor (TCR). These findings support the role of Rin2 as a novel general negative regulator/effector of Ras and Ras-dependent signaling pathways in diverse cell types which have been activated via distinct cell surface receptors (Figure 2). In addition, the enhancement or inhibition of Rin2 expression or function, in certain

settings, resulting in the reduction or enhancement, respectively, of Ras-dependent signaling in these settings, may have benefit in diverse clinical problems, some examples of which are shown in Figure 2.

As also described herein, the SY-A (Rin2) clone has been used as a probe to screen a HT-29 human adenocarcinoma cDNA library. Two overlapping cDNA clones (HRIN2-1B1 (1.3 kb) and HRIN2-2B1 (2.9 kb)) have been identified which are highly homologous to that of the mouse Rin2 clone and which together represent the full-length human Rin2 (Rabex-5) cDNA sequence. The orientation of these overlapping clones is shown in Figure 9. The nucleotide sequence of each clone is shown in Figures 10A-B and 11A-C, respectively.

Accordingly, this invention pertains to isolated Rin2 protein, which down-regulates the functional responses elicited by Ras-dependent signaling pathways. In one embodiment, Rin2 down-regulates the functional responses elicited by FcεRI aggregation in mast cells (e.g., mammalian mast cells), including down-regulating the amounts of preformed mediator (serotonin) and cytokine (IL-6) released from these cells upon FcεRI-dependent stimulation. In another embodiment, Rin2 down-regulates functional responses in mast cells stimulated via activation of their major growth factor receptor (i.e., c-kit). In another embodiment, Rin2 inhibits cell proliferation in PC12 adrenal pheochromocytoma cells stimulated through the nerve growth factor receptor (i.e., TrkA). In a further embodiment, Rin2 inhibits functional responses in T cells activated through the T cell receptor.

In one embodiment, isolated Rin2 protein has the amino acid sequence of SEQ ID NO: 2. In one embodiment, the Rin2 protein is a portion of SEQ ID NO: 2 which is sufficient for Rin2 activity, e.g., binding or enzymatic activity. Also included within the meaning of Rin2 protein or polypeptide are polypeptides which are less than the amino acid sequence of SEQ ID NO: 2 and have Rin2 activity, as well as proteins whose amino acid sequence is substantially similar to that of SEQ ID NO: 2 and have Rin2 activity.

In another embodiment, Rin2 protein is a derivative possessing substantial sequence identity with the endogenous Rin2 protein. In particular embodiments, the Rin2 protein is purified to homogeneity or is substantially free of other proteins.

The invention also pertains to an isolated nucleic acid molecule or nucleotide sequence (*rin2*) which encodes Rin2 protein or polypeptide, or a portion of SEQ ID NO: 2 which is sufficient for Rin2 activity. In one embodiment, the encoded Rin2 protein is a derivative possessing substantial sequence identity with the endogenous Rin2 protein. In a particular embodiment, the isolated nucleic acid molecule encodes Rin2 protein with the same amino acid sequence as endogenous Rin2 protein. In another embodiment, the isolated nucleic acid molecule has the same nucleotide sequence as the endogenous gene encoding Rin2 protein. In one embodiment, the isolated nucleic acid molecule has the nucleotide sequence of SEQ ID NO: 1. In another embodiment, the isolated nucleic acid molecule comprises the nucleotide sequence of SEQ ID NO: 12 and/or the nucleotide sequence of SEQ ID NO: 13. In a further

embodiment, the isolated nucleic acid molecule comprises the nucleotide sequence of SEQ ID NO: 14 and/or the nucleotide sequence of SEQ ID NO: 15. In one embodiment the isolated *rin2* nucleic acid molecule comprises the nucleotide sequence of one or more of SEQ ID NOS: 12, 13, 14 and 15.

The invention also relates to DNA constructs comprising the nucleic acid molecules described herein, operatively linked to a regulatory sequence, and to recombinant host cells, such as bacterial cells, fungal cells, plant cells, insect cells and mammalian cells, comprising the nucleic acid molecules described herein operatively linked to a regulatory sequence. The invention also relates to a method for preparing a Rin2 polypeptide, comprising culturing a recombinant host cell described herein.

The invention also pertains to an antibody, or an antigen-binding fragment thereof, which selectively binds to Rin2 protein, or a portion of SEQ ID NO: 2 which is sufficient for Rin2 activity; in a particular embodiment, the antibody is a monoclonal antibody. The invention also relates to a method for assaying the presence of Rin2 protein in a cell, e.g., in a tissue sample, comprising contacting said cell with an antibody which specifically binds to Rin2 protein.

The present invention also relates to an assay for identifying agents which alter the activity of Rin2 protein. For example, a cell population, e.g., a mast cell population, containing Rin2 protein can be activated with a stimulus which activates at least one Ras-dependent pathway in the cell in the presence of an

agent to be tested, and the level of Rin2 activity can be assessed.

The invention further relates to methods of inhibiting the functional responses (e.g., proliferation, functional activation) elicited by activation of Ras-dependent signaling pathways in cells, comprising contacting the cells with an agent which enhances or mimics the activity of Rin2 protein. The invention also relates to a method of treating a mammal in need thereof to inhibit functional responses elicited by activation of Ras-dependent signaling pathways, comprising administering to a mammal an agent which enhances or mimics the activity of native Rin2 protein.

The invention further relates to methods of inhibiting the functional responses elicited by FcεRI aggregation, particularly in mast cells, comprising contacting a mast cell population with an agent which enhances or mimics the activity of native Rin2 protein. The invention relates to a method of treating a mammal in need thereof to inhibit the functional response elicited by FcεRI aggregation, comprising administering to a mammal an agent which enhances the activity of native Rin2 protein. For example, the invention relates to methods of inhibiting IgE and antigen-dependent release of mediators from mast cells. Such methods can be used to inhibit mediator release from mast cells and other effector cells that express FcεRI, such as basophils, monocytes/macrophages, dendritic cells, Langerhans' cells and eosinophils, thereby ameliorating disorders such as asthma and allergic diseases (e.g., hay fever and atopic eczema).

The invention further relates to methods of enhancing functional responses (e.g., proliferation, functional activation) elicited by activation of Ras-dependent signaling pathways in cells, comprising contacting the cells with an agent which inhibits the activity of Rin2 protein. The invention also relates to a method of treating a mammal in need thereof to enhance functional responses elicited by activation of Ras-dependent signaling pathways, comprising administering to a mammal an agent which inhibits the activity of Rin2 protein.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a schematic illustration of the pathways of Ras activation and Ras-dependent changes in cell proliferation, differentiation and function.

Figure 2 is an illustration of alteration (increase or decrease) in Rin2 expression or activity and the corresponding effect on Ras-dependent signaling. Also illustrated are examples of instances in which it may be clinically desirable to alter Rin2 expression or activity and thereby alter, positively or negatively, Ras-dependent signaling.

Figure 3 is the cDNA sequence (SEQ ID NO: 1) and the predicted amino acid sequence (SEQ ID NO: 2) of the SY-A clone (Rin2). The predicted open reading frame begins at nucleotide 60 and ends at nucleotide 1535.

Figure 4 shows an alignment of the amino acid sequences of Rin2 with the GTPase binding homology (GBH) domains of Vps9p, Rin1, and JC265. The GBH domains of Vps9p, Rin1, and JC265 and the GBH motifs I, II, and III illustrated here represent those described in Burd *et*

al. (*Mol. Cell. Biol. 16*:2369-2377 (1996)). Proteins are identified on the left, and the position of the amino acid on each line is indicated. Identical amino acids or conservative substitutions present in three of four proteins are shaded. Conservative substitutions for the alignment were assigned as follows: V, L, I, and M; K, R, and H; F, Y, and W; D and E; N and Q; S and T; and G and A.

Figure 5 is a graph of the kinetics of Erk-MAP kinase activation induced by FcεRI aggregation in C1.MC/C57.1 mast cells transfected with the control vector (pBK-CMV) or in C1.MC/C57.1 cells transfected with the antisense expression vector (pBK-CMV-SYA-AS).

Figure 6 is a graph of the release of ^{3}H-serotonin induced by FcεRI aggregation in C1.MC/C57.1 mast cells transfected with the control vector (pBK-CMV) or in C1.MC/C57.1 cells transfected with the antisense expression vector (pBK-CMV-SYA-AS). Release of serotonin, assessed as percentage of specific release of [^{3}H]-5HT, was measured 10 minutes after challenge. An asterisk indicates $P< 0.05$ versus corresponding values for cells transfected with the control pBK-CMV vector (n=4 to 5 per point).

Figures 7A and 7B are graphs of the kinetics of IL-6 release by mast cells. Figure 7A illustrates the kinetics of IL-6 release induced by FcεRI aggregation in C1.MC/C57.1 mast cells transfected with the control vector (pBK-CMV) or in C1.MC/C57.1 cells transfected with the antisense expression vector (pBK-CMV-SYA-AS). Transfected mouse mast cells sensitized with anti-DNP IgE monoclonal antibody (mAb) for 2 hours were challenged with DNP-HSA. Figure 7B illustrates IL-6

release from transfected C1.MC/C57.1 mast cells 6 hours after stimulation by different concentrations of DNP-HSA. An asterisk indicates $P< 0.05$ versus corresponding values for cells transfected with the control pBK-CMV vector (n=4 to 5 per point).

Figures 8A and 8B are graphs illustrating that expression of antisense *rin2* mRNA enhances cell proliferation in PC12 cells, a rat adrenal pheochromocytoma cell line. Rate of proliferation of PC12 cells transfected with *rin2* antisense expression vector (pBK-CMV-SYA-AS), or PC12 cells transfected with the control CMV vector (pBK-CMV), or untransfected PC12 cells, was measured by assessing [^{3}H]thymidine incorporation in cells which had been incubated with [^{3}H] thymidine for 3 hours after the cells had been cultured overnight in normal culture medium (Figure 8A), or in medium containing nerve growth factor (NGF) of indicated concentrations and 0.5% FCS (+NGF; Figure 8B). An asterisk indicates $P< 0.01$ versus corresponding values for untransfected PC12 cells or PC12 cells that had been transfected with the control pBK-CMV vector (n=4 per point).

Figure 9 shows the orientation of overlapping human cDNA clones HRIN2-1B1 and HRIN2-2B1 relative to one another.

Figures 10A-B show an alignment of murine Rin2 with the partial nucleotide sequence of human cDNA clone HRIN2-1B1 from nucleotide 51 through nucleotide 405 (SEQ ID NO: 12) and from nucleotide 885 through nucleotide 1144 (SEQ ID NO: 13). The sequence numbering is the nucleotide number in the murine Rin2 sequence.

Figures 11A-C show an alignment of murine Rin2 with the partial nucleotide sequence of human cDNA clone HRIN2-2B1 from nucleotide 532 to nucleotide 1276 (SEQ ID NO: 14)(Figures 11A-B) and a portion of the nucleotide sequence of the 3' end (SEQ ID NO: 15) (Figure 11C). In Figures 11A-B, the sequence numbering is the nucleotide number in the murine Rin2 sequence.

Figure 12 shows the interactions of murine Rin2 with murine H-Ras protein or yeast Ras2p protein in the yeast two-hybrid assay. Different segments of the murine Rin2 protein (shaded areas are those of greatest sequence homology to Vps9p, JC265 and Rin1; black areas are GBH motifs) were expressed as GAL4-activation domain fusion proteins, whereas the murine H-Ras and yeast Ras2p proteins were expressed as DNA-binding fusion proteins. Positive interactions were assessed with both a qualitative colony-lift β-galactosidase filter assay using X-gal as the substrate (colony color) and a quantitative liquid culture β-galactosidase assay using chlorophenol red-b-D-galactopyranoside (CPRG) as the substrate (relative β-gal units).

Figures 13A-B are graphs showing that *rin2* antisense transfectants release normal levels of preformed mediators but elevated levels of cytokines in response to FcεRI aggregation. Figure 13A shows the release of β-hexosaminidase (β-hex) or IL-6 induced by FcεRI aggregation in C1.MC/C57.1 mast cells transfected with the *rin2* antisense expression vector (AS) or the control CMV vector (control). Transfectant cells sensitized with anti-DNP IgE mAb for 2 hours were challenged with DNP-HSA. Specific release of β-hexosaminidase (background release was ≤ 6%) was

measured 15 minutes after antigen challenge, whereas IL-6 release was measured by ELISA assay 6 hours after antigen challenge. The numbers over the columns indicate the numbers of stable lines of transfectants established from two independent transfections and assayed in this experiment. An asterisk indicates $p=0.03$ vs. control by unpaired Student's *t* test. Figure 13B shows the kinetics of IL-6 release induced by FcεRI aggregation in C1.MC/C57.1 mast cells transfected with the antisense (AS) or control vector. An asterisk indicates $p<0.01$ versus corresponding values for the control transfectant cells.

Figure 14 is a graph showing the kinetics of JNK activation induced by FcεRI aggregation in C1.MC/C57.1 cells transfected with the *rin2* antisense expression or the control vector. Findings are representative of those obtained with 3-4 different antisense and control transfectants.

Figure 15 is a graph showing the kinetics of p38 MAP kinase activation induced by FcεRI aggregation in C1.MC/C57.1 cells transfected with the *rin2* antisense expression vector or the control vector. Findings are representative of those obtained with 3-4 different antisense and control transfectants.

DETAILED DESCRIPTION OF THE INVENTION

In order to elucidate specific signaling pathways involved in FcεRI-mediated mast cell activation, the technique of mRNA differential display was used to isolate cDNAs of genes that were differentially expressed in mast cells stimulated through the FcεRI. Work described herein identified the cDNA of a novel

gene, *rin2*, whose expression is rapidly increased in mammalian mast cells activated through FcεRI. The *rin2* gene product shares significant homology with members of a class of Ras-binding proteins, such as Rin1 and JC265 (Colicelli *et al.*, *Proc. Natl. Acad. Sci. USA* *88*:2913-2917 (1991); Han and Colicelli, *Mol. Cell. Biol.* *15*:1318-1323 (1995)), which have been shown to negatively regulate Ras-mediated signaling in *Saccharomyces (S.) cerevisiae*, and Vps9p (Burd *et al.*, *Mol. Cell. Biol.* *16*:2369-2377 (1996)), which is required for vacuolar protein sorting. As shown herein, inhibition of Rin2 expression potentiates both the intracellular signaling responses and the cellular secretory responses that are induced by FcεRI aggregation. The work described herein supports the role of Rin2 as a novel negative regulator/effector of Ras-dependent signaling pathways in mast cells.

The murine *rin2* gene encodes a protein of predicted molecular mass of 56.9 kDa which shares significant homology to a class of Ras-binding proteins represented by JC265, Rin1 and Vps9p. Rin1 and JC265 were identified by virtue of their ability to suppress an activated Ras mutant allele in *S. cerevisiae*, indicating that they can negatively regulate Ras-induced signaling responses (Colicelli *et al.*, *Proc. Natl. Acad. Sci. USA* *88*:2913-2917 (1991)). In addition, recent studies have shown that Rin1 interacts directly with the Ras protein in a manner similar to that observed with Raf1 and yeast adenylyl cyclase, which are known downstream effectors of Ras (Han and Colicelli, *Mol. Cell. Biol.* *15*:1318-1323 (1995)). Thus, Rin1 binds primarily to the effector domain of Ras and functions as a downstream effector of

Ras-GTP. On the other hand, the gene encoding Vps9p was isolated in yeast by complementation of a vacuolar protein sorting defect associated with the *vps9* mutant (Burd *et al.*, *Mol. Cell. Biol. 16*:2369-2377 (1996)). Thus, Vps9p is a 53 kDa protein required for the proper sorting of several vacuolar proteins and may function as part of the vacuolar protein sorting machinery in *S. cerevisiae*.

The highest homology shared between JC265, Rin1, Vps9p, and Rin2 is found in the GTPase binding homology (GBH) domain regions in which three putative GBH motifs have been described (Burd *et al.*, *Mol. Cell. Biol. 16*:2369-2377 (1996)). In particular, the GBH motif II is situated within the regions of JC265 and Rin1 that have been previously found to be shared with GAP and GAP-like proteins such as yeast IRA1 and IRA2, NF1, and sar1 (Colicelli *et al.*, *Proc. Natl. Acad. Sci. USA 88*:2913-2917 (1991)). Furthermore, an FLP consensus motif can be detected within the GBH motif II regions of JC265, Rin1, Vps9p, and Rin2, and this FLP motif aligns well with the FLR consensus motif (residues 901-903) present in the block 3A region that is common to all GAP proteins (Scheffzek *et al.*, *Nature 384*:591-596 (1996)). Mutation analysis has shown that the phenylalanine present at position 901 is required for core stabilization of the Ras protein and that leucine present at position 902 is important in the functional interaction between GAP and Ras (Miao *et al.*, *J. Biol. Chem. 271*:15322-15329 (1996); Hettich and Marshall, *Cancer Res. 54*:5438-5444 (1994); Brownbridge et al., *J. Biol. Chem. 268*:10914-10919 (1993)). The presence of the FLP consensus motif in the Rin2 amino acid sequence

provides additional support for the role of Rin2 as a regulator of Ras.

As also described herein, the SY-A (Rin2) clone has been used as a probe to screen a HT-29 human adenocarcinoma cDNA library. Two overlapping cDNA clones (HRIN2-1B1 (1.3 kb) and HRIN2-2B1 (2.9 kb)) have been identified which are highly homologous to that of the mouse Rin2 clone and which together represent the full-length human Rin2 (Rabex-5) cDNA sequence. The orientation of these overlapping clones is shown in Figure 9. The nucleotide sequence of each clone is shown in Figures 10A-B and 11A-C, respectively. The determination of the complete sequence of these clones can be carried out by the skilled artisan using methods well known in the art.

The invention pertains to an isolated nucleotide sequence encoding Rin2 protein. In one embodiment the isolated nucleotide sequence is a mammalian nucleotide sequence. In a particular embodiment the nucleotide sequence is a murine or human nucleotide sequence. As appropriate, nucleic acid molecules of the present invention can be RNA, for example, mRNA, or DNA, such as cDNA and genomic DNA. DNA molecules can be double-stranded or single-stranded; single stranded RNA or DNA can be either the coding (sense) strand or the non-coding (antisense) strand. The nucleotide sequence can encode a portion of the amino acid sequence of the Rin2 protein; alternatively, the nucleotide sequence can include at least a portion of the Rin2 amino acid coding sequence along with additional non-coding sequences, such as introns and non-coding 3' and 5' sequences (including regulatory sequences, for example).

Additionally, the nucleotide sequence can be fused to a marker sequence, for example, a sequence which encodes a polypeptide to assist in isolation or purification of the Rin2 protein. Such sequences include, but are not limited to, those which encode a glutathione-S-transfeRase (GST) fusion protein and those which encode a hemaglutin A (HA) peptide marker from influenza.

As used herein, "isolated" is intended to mean that the material in question exists in a physical milieu distinct from that in which it occurs in nature and/or has been completely or partially purified from other transcribed sequences (as in a cDNA or RNA library). Thus, an isolated gene or nucleotide sequence can include a gene or nucleotide sequence which is synthesized chemically or by recombinant means. Recombinant DNA contained in a vector is included in the definition of "isolated" as used herein. Also, isolated nucleotide sequences include recombinant DNA molecules in heterologous host cells, as well as partially or substantially purified DNA molecules in solution. *In vivo* and *in vitro* RNA transcripts of the DNA molecules of the present invention are also encompassed by the term "isolated" nucleotide sequences. Such isolated nucleotide sequences are useful in the manufacture of the encoded protein, as probes for isolating homologous sequences (e.g., from other mammalian species), for gene mapping (e.g., by *in situ* hybridization with chromosomes), or for detecting expression of the *rin2* gene in tissue (e.g., human tissue), such as by Northern blot analysis.

The invention relates to nucleic acid molecules comprising the nucleotide sequence of SEQ ID NO: 1 or

the complement of SEQ ID NO: 1. The invention also relates to nucleic acid molecules comprising the nucleotide sequence of SEQ ID NO: 12 and/or SEQ ID NO: 13. That is, the nucleic acid molecule can comprise SEQ ID NO: 12, SEQ ID NO: 13 or both SEQ ID NOS: 12 and 13. The invention further relates to a nucleic acid molecule comprising the nucleotide sequence of SEQ ID NO: 14 and/or SEQ ID NO: 15. That is, the nucleic acid molecule can comprise SEQ ID NO: 14, SEQ ID NO: 15 or both SEQ ID NOS: 14 and 15. In a particular embodiment, the nucleic acid molecule comprises one or more nucleotide sequences selected from the group consisting of SEQ ID NOS: 12, 13, 14 and 15.

The present invention also pertains to nucleotide sequences which are not necessarily found in nature but which encode Rin2 protein. Thus, DNA molecules which comprise a sequence which is different from the naturally-occurring nucleotide sequence but which, due to the degeneracy of the genetic code, encode Rin2 protein are the subject of this invention. The invention also encompasses variations of the nucleotide sequences of the invention, such as those encoding portions, analogues or derivatives of Rin2 protein. Such variations can be naturally-occurring, such as in the case of allelic variation, or non-naturally-occurring, such as those induced by various mutagens and mutagenic processes. Included variations include, but are not limited to, addition, deletion and substitution of one or more nucleotides which can result in conservative or non-conservative amino acid changes, including additions and deletions. Preferably, the nucleotide or amino acid variations are silent or

conserved, respectively; that is, they do not alter the characteristics or activity of Rin2 protein.

The invention described herein also relates to fragments of the isolated nucleic acid molecules described above. The term "fragment" is intended to encompass a portion of a nucleotide sequence described herein which is from at least about 25 contiguous nucleotides to at least about 50 contiguous nucleotides or longer in length; such fragments are useful as probes, e.g., for diagnostic methods and also as primers, and can encode functional portions of the Rin2 polypeptide. Particularly preferred primers and probes selectively hybridize to the nucleic acid molecule encoding Rin2 protein described herein. For example, fragments which encode antigenic regions of the Rin2 protein described herein are useful.

The invention also pertains to nucleotide sequences which hybridize under medium stringency hybridization conditions (e.g., for selective hybridization) to a nucleotide sequence described herein. Appropriate stringency conditions which are considered "medium stringency" are known to those skilled in the art or can be found in standard texts such as *Current Protocols in Molecular Biology*, John Wiley & Sons, N.Y. (1989), 6.3.1-6.3.6. Preferred hybridization conditions allow for specific hybridization of a nucleotide sequence to a nucleotide sequence described herein. Such hybridizable nucleotide sequences are useful as probes and primers for diagnostic applications.

The invention pertains to nucleotide sequences which have a substantial identity with the nucleotide sequence of SEQ ID NO: 1; particularly preferred are

nucleotide sequences which have at least about 75%, preferably at least about 80%, more preferably at least about 85%, and more preferably at least about 95% identity with nucleotide sequences described herein. Particularly preferred in this instance are nucleotide sequences encoding polypeptides having at least one activity of the novel Rin2 protein described herein, or which hybridize with characteristic sequences of *rin2* and can therefore be used as probes or primers. For example, preferred nucleotide sequences encoding a polypeptide having the same or similar biological activity as the Rin2 protein, and nucleotide sequences encoding a polypeptide with the same or similar immunogenic or antigenic properties as the Rin2 protein are within the scope of the invention. As used herein, activities of the Rin2 protein include, but are not limited to, catalytic activity, binding function, antigenic function and oligomerization function.

This invention also pertains to an isolated protein or polypeptide which is a novel Rin2 protein, such as the protein or polypeptide encoded by any one or more of SEQ ID NOS: 1, 12, 13 and 14. Rin2 protein down-regulates the functional responses elicited by FcεRI aggregation in mast cells, including down-regulating the amounts of preformed mediator (serotonin) and cytokine (IL-6) released from these cells upon FcεRI-dependent stimulation. It may also down-regulate responses elicited at other types of receptors (such as TrkA receptors for NGF, c-kit receptors for SCF, etc.) in response to receptor interaction with ligand or due to auto-activation of the receptors (e.g., by activating mutations).

For example, an isolated protein of the invention may be substantially isolated with respect to the complex cellular milieu in which it naturally occurs. In some instances, the isolated material will form part of a composition (for example, a crude extract containing other substances), buffer system or reagent mix. In other circumstances, the material may be purified to essential homogeneity, for example as determined by PAGE or column chromatography such as HPLC. According to the invention, the amino acid sequence of the polypeptide can be that of the naturally-occurring protein or can comprise alterations therein. Such alterations include conservative or non-conservative amino acid substitutions, additions and deletions of one or more amino acids; however, such alterations should preserve at least one activity of the Rin2 protein, i.e., the altered or mutant protein should be an active derivative of the naturally-occurring protein. For example, the mutation(s) can preferably preserve the three dimensional configuration of the binding and/or catalytic site of the native protein. The presence or absence of Rin2 activity can be determined by various functional assays, as described herein. Moreover, amino acids which are essential for the function of the Rin2 protein can be identified by methods known in the art. Particularly useful methods include identification of conserved amino acids in the family or subfamily of Ras-interference or Ras-inhibiting proteins, site-directed mutagenesis and alanine-scanning mutagenesis (for example, Cunningham and Wells, *Science 244*:1081-1085 (1989)), crystallization and nuclear magnetic resonance. The

altered polypeptides produced by these methods can be tested for particular biologic activities, including immunogenicity and antigenicity.

Specifically, appropriate amino acid alterations can be made on the basis of several criteria, including hydrophobicity, basic or acidic character, charge, polarity, size, the presence or absence of a functional group (e.g., -SH or a glycosylation site), and aromatic character. Assignment of various amino acids to similar groups based on the properties above will be readily apparent to the skilled artisan (see, for example, Figure 4); further appropriate amino acid changes can also be found in Bowie *et al.* (*Science 247*:1306-1310(1990)).

The Rin2 polypeptide can also be a fusion protein comprising all or a portion of the Rin2 amino acid sequence fused to an additional component. Additional components, such as radioisotopes and antigenic tags, can be selected to assist in the isolation or purification of the polypeptide or to extend the half life of the polypeptide; for example, a hexahistidine tag permits ready purification by nickel chromatography.

Also included in the invention are novel polypeptides which are at least about 40% similar to the Rin2 protein described herein. However, polypeptides exhibiting lower levels of identity are also useful, particularly if they exhibit high, e.g., at least about 40%, similarity over one or more particular domains of the protein. For example, polypeptides sharing high degrees of identity or similarity over domains necessary for particular activities, including binding and enzymatic activity, are included herein. As used

herein, "similar" amino acids are intended to mean identical amino acids or conserved substitutions of amino acids.

Polypeptides described herein can be isolated from naturally-occurring sources, chemically synthesized or recombinantly produced. Polypeptides or proteins of the present invention can be used as a molecular weight marker on SDS-PAGE gels or on molecular sieve gel filtration columns using art-recognized methods.

The invention also provides expression vectors containing a nucleic acid sequence encoding a polypeptide which is a Rin2 polypeptide, operably linked to at least one regulatory sequence. Many such vectors are commercially available, and other suitable vectors can be readily prepared by the skilled artisan. "Operably linked" is intended to meant that the nucleotide sequence is linked to a regulatory sequence in a manner which allows expression of the nucleic acid sequence in a host cell. Regulatory sequences are art-recognized and are selected to produce a polypeptide which is Rin2. Accordingly, the term "regulatory sequence" includes promoters, enhancers, and other expression control elements which are described in Goeddel, *Gene Expression Technology: Methods in Enzymology 185*, Academic Press, San Diego, CA (1990). For example, the native regulatory sequences or regulatory sequences native to a transformed host cell can be employed. It should be understood that the design of the expression vector may depend on such factors as the choice of the host cell to be transformed and/or the type of protein desired to be expressed. For instance, the polypeptides of the present invention can

be produced by ligating the cloned gene, or a portion thereof, into a vector suitable for expression in either prokaryotic cells, eukaryotic cells or both (see, for example, Broach, *et al.*, *Experimental Manipulation of Gene Expression*, ed. M. Inouye (Academic Press, 1983) p. 83; *Molecular Cloning: A Laboratory Manual*, 2nd Ed., ed. Sambrook *et al.* (Cold Spring Harbor Laboratory Press, 1989) Chapters 16 and 17). Typically, expression constructs will contain one or more selectable markers, including, but not limited to, the gene that encodes dihydrofolate reductase and the genes that confer resistance to geneticin, neomycin, tetracycline, ampicillin, chloramphenicol, kanamycin and streptomycin resistance.

Prokaryotic and eukaryotic host cells transfected by the described vectors are also provided by this invention. For instance, cells which can be transfected with the vectors of the present invention include, but are not limited to, bacterial cells such as *E. coli* (e.g., *E. coli* K12 strains), *Streptomyces*, *Pseudomonas*, *Serratia marcescens* and *Salmonella typhimurium*, insect cells (baculovirus), including *Drosophila*, fungal cells, such as yeast cells, plant cells and mammalian cells, such as mast cells, PC12 cells, EL-4 T cells or other T cells, Chinese hamster ovary cells (CHO) and COS cells.

Thus, a nucleotide sequence derived from the cloning of Rin2 described herein can be used to produce a recombinant form of the protein via microbial or eukaryotic cellular processes. Ligating the polynucleotide sequence into a gene construct, such as an expression vector, and transforming or transfecting into hosts, either eukaryotic (yeast, avian, insect,

plant or mammalian) or prokaryotic (bacterial cells), are standard procedures used in producing other well known proteins. Similar procedures, or modifications thereof, can be employed to prepare recombinant proteins according to the present invention by microbial means or tissue-culture technology. Accordingly, the invention pertains to the production of Rin2 proteins or polypeptides by recombinant technology.

The proteins and polypeptides of the present invention can be isolated or purified (e.g., to homogeneity) from recombinant cell culture by a variety of processes. These include, but are not limited to, anion or cation exchange chromatography, ethanol precipitation, affinity chromatography and high performance liquid chromatography (HPLC). The particular method used will depend upon the properties of the polypeptide and the selection of the host cell; appropriate methods will be readily apparent to those skilled in the art.

The present invention also relates to antibodies which bind a polypeptide which is Rin2. For instance, polyclonal and monoclonal antibodies, including non-human and human antibodies, humanized antibodies, chimeric antibodies and antigen-binding fragments thereof (*Current Protocols in Immunology*, John Wiley & Sons, N.Y. (1994); EP Application 173,494 (Morrison); International Patent Application WO86/01533 (Neuberger); and U.S. Patent No. 5,225,539 (Winters)) which bind to the described protein are within the scope of the invention. A mammal, such as a mouse, rat, hamster or rabbit, can be immunized with an immunogenic form of Rin2 (e.g., Rin2 or a peptide comprising an antigenic

fragment of Rin2 which is capable of eliciting an antibody response). Techniques for conferring immunogenicity on a protein or peptide include conjugation to carriers or other techniques well known in the art. The protein or polypeptide can be administered in the presence of an adjuvant. The progress of immunization can be monitored by detection of antibody titers in plasma or serum. Standard ELISA or other immunoassays can be used with the immunogen as antigen to assess the levels of antibody.

Following immunization, anti-peptide antisera can be obtained, and if desired, polyclonal antibodies can be isolated from the serum. Monoclonal antibodies can also be produced by standard techniques which are well known in the art (Kohler and Milstein, *Nature 256*:495-497 (1975); Kozbar et al., *Immunology Today* 4:72 (1983); and Cole et al., *Monoclonal Antibodies and Cancer Therapy*, Alan R. Liss, Inc., pp. 77-96 (1985)). The term "antibody" as used herein is intended to include fragments thereof, such as Fab and $F(ab)_2$. Antibodies described herein can be used to inhibit the activity of Rin2 described herein, particularly *in vitro* and in cell extracts, using methods known in the art. Additionally, such antibodies, in conjunction with a label, such as a radioactive label, can be used to assay for the presence of the expressed protein in a cell from, e.g., a tissue sample, and can be used in an immunoabsorption process, such as an ELISA, to isolate the Rin2 protein. Tissue samples which can be assayed include human tissues, e.g., differentiated and non-differentiated cells. Examples include, but are not limited to, heart, lung, skin, skeletal muscle, bone marrow, thymus, lymph node,

spleen, kidney, liver, brain, pancreas, gastrointestinal tract, gonads (testes, ovaries), fibroblasts and epithelium.

The present invention also pertains to pharmaceutical compositions comprising polypeptides described herein. For instance, a polypeptide or protein, or prodrug thereof, of the present invention can be formulated with a physiologically acceptable medium to prepare a pharmaceutical composition. The particular physiological medium may include, but is not limited to, water, buffered saline, polyols (e.g., glycerol, propylene glycol, liquid polyethylene glycol) and dextrose solutions. The optimum concentration of the active ingredient(s) in the chosen medium can be determined empirically, according to well known procedures, and will depend on the ultimate pharmaceutical formulation desired. Methods of introduction of exogenous peptides at the site of treatment include, but are not limited to, intradermal, intramuscular, intraperitoneal, intravenous, subcutaneous, oral, intranasal intrarectal, intravaginal, and aerosol (for administration to the respiratory tract). Other suitable methods of introduction can also include gene therapy, rechargeable or biodegradable devices and slow release polymeric devices. The pharmaceutical compositions of this invention can also be administered as part of a combinatorial therapy with other agents.

While the intracellular events which lead to the activation of cell proliferation, differentiation, or functional responses (e.g., FceRI-dependent mast cell mediator secretion) have been investigated in great

detail, equally as interesting are the pathways which can suppress such responses. For example, recent evidence indicates that certain cell surface receptors, such as FcγRIIb or gp149 in mast cells, can mediate signals which can down-regulate the cell activation signals which are transduced by other cell surface receptors, including the mast cell FcεRI (Scharenberg and Kinet, *Cell 87*:961-964 (1996)). However, the work described herein is directed to searching for intracellular molecules which function to down-regulate the signaling pathways in which Ras plays a role, including those which govern the secretion of mast cell mediators or the proliferative responses of neuronal cells or other cell types. Ras plays a pivotal role in signaling pathways which mediate diverse cellular responses to a spectrum of receptor-ligand interactions (Bourne *et al.*, *Nature 348*:125-132 (1990); Marshall, *FASEB J. 9*:1311-1318 (1995)). Recent studies have shown that the functional activation of mast cells which is induced by the aggregation of FcεRI by IgE and specific antigen is associated with the activation of Ras and its effector pathways (Beaven and Baumgartner, *Curr. Opin. Immunol. 8*:766-772 (1996); Jabril-Cuenod *et al.*, *J. Biol. Chem. 271*:16268-16272 (1996)).

FcεRI-mediated Ras activation in mast cells is accompanied by activation of the Shc-Grb2-Sos pathway, which precedes Ras activation, as well as the Ras effector pathway of Raf-1/Erk-MAP kinases (Jabril-Cuenod *et al.*, *J. Biol. Chem. 271*:16268-16272 (1996)). Recent studies with rat RBL-2H3 mast cells have demonstrated that FcεRI-induced Ras activation results in a transcription activation mediated by Elk-1 and NFAT

(Turner and Cantrell, *J. Exp. Med. 185*:43-53 (1997)). The Elk-1 transcription factor exerts its effects by interacting with the serum response element, which is a regulatory component of early response genes such as *c-fos* (Treisman, *Curr. Opin. Genet. Dev.* 4:96-101 (1994)), whereas members of the NFAT transcription factor family form regulatory complexes with inducible nuclear factor such as Fos or Jun (Rao, *Immunol. Today 15*:274-281 (1994)). Such complexes interact with cytokine gene promoters and activate transcriptions of genes for IL-2, IL-4, GM-CSF, and TNF-α (Rao, *Immunol. Today 15*:274-281 (1994)). In mast cells, NFAT is implicated in the regulation of transcription of the IL-4 gene (Weiss *et al.*, *Mol. Cell. Biol. 16*:228-235 (1996)). Interestingly, the activation of the Raf-1/MEK/Erk cascade is necessary and sufficient for the activation of Elk-1 in mast cells, whereas the induction of NFAT by FcεRI stimulation requires the activity of Rac-1 (Turner and Cantrell, *J. Exp. Med. 185*:43-53 (1997)). Earlier studies have shown that Rin1 interacts directly with Ras in a manner that is characteristic of Raf-1, suggesting that Rin1 is a negative downstream effector of Ras (Han and Colicelli, *Mol. Cell. Biol. 15*:1318-1323 (1995)).

As shown herein, reduced expression of Rin2 can dramatically potentiate the downstream signaling responses mediated by Ras. In mouse mast cells transfected with the *rin2* antisense expression vector, the induction of early-response gene expression and the activation of MAP kinase activity in response to FcεRI crosslinking are significantly augmented, as compared with those observed in mast cells transfected with a

control vector. These results support a role of Rin2 as a negative effector/regulator of Ras. Furthermore, reduced expression of Rin2 can markedly enhance the functional responses elicited by FcεRI aggregation in mouse mast cells; the expression of antisense *rin2* in mouse mast cells can significantly increase the amounts of preformed mediators (e.g., 5-HT) and cytokine (e.g., IL-6) released from these cells upon FcεRI-dependent stimulation. These data indicate that Rin2 can negatively regulate the Ras-mediated signaling responses in mast cells that lead to both the release of preformed mediators and the transcription of cytokine genes and the secretion of the corresponding products. Stimulation of mast cells by IgE and antigen results in Ras activation followed by a rapid and transient induction of *rin2* mRNA expression. Rin2 appears to exert its effects by down-regulating the signaling and functional responses elicited by FcεRI aggregation. Thus, during the period subsequent to mast cell activation, Rin2 may function as a cellular switch in turning off ongoing signaling initiated by Ras activation. In mast cells, such a regulatory process may be important as a feedback mechanism to restore the perturbed cellular environment to its normal basal physiological state after the cessation of cell activation.

Furthermore, *rin2* mRNA expression is significantly elevated in mouse mast cells stimulated with stem cell factor (SCF), with a kinetics of activation that is very similar to that which is observed in mast cells after FcεRI-dependent stimulation. SCF, the cognate ligand for the c-kit tyrosine kinase receptor, is a major mast

cell growth factor which has been shown to be critical in the maturation and development/function of rodent and human mast cells (Galli *et al.*, *Adv. Immunol.* *55*:1-96 (1994)). The interaction of SCF with the c-kit tyrosine kinase receptor has been shown to induce an activation of Ras and its effector pathways via the activation of the Shc-Grb2-Sos signaling pathway (Duronio *et al.*, *Proc. Natl. Acad. Sci. USA* *89*:1587-1591 (1992); Tauchi *et al.*, *J. Exp. Med.* *179*:167-175 (1994)).

As shown herein, nerve growth factor (NGF) can also elicit a rapid and sustained induction of expression of *rin2* mRNA transcript through it receptor. NGF has been shown to induce neuronal differentiation and cessation of cell proliferation in PC12 cells via its interaction with TrkA receptor tyrosine kinase (Barbacid, *J. Neurobiol.* *25*:1386-1403 (1994); Bothwell, *Ann. Rev. Neurosci.* *18*:223-253 (1995)). PC12 cells are a commonly used *in vitro* model of neuronal development and function (Greene and Tischler, *Proc. Natl. Acad. Sci. USA* *73*:2424-2428 (1976)). Previous studies have indicated that NGF stimulation of TrkA receptors in PC12 cells leads to Ras activation and the activation of the Raf-1/MEK/MAP kinases effector pathway (Thomas *et al.*, *Cell* *68*:1031-1040 (1992); Wood *et al.*, *Cell* *68*:1041-50 (1992)). Results presented herein show that PC12 cells which have been transfected with the pBK-CMV-SYA-AS vector and therefore have reduced expression of Rin2 can still differentiate in response to NGF stimulation. However, these transfected cells exhibit a higher rate of cell proliferation than that detected in control PC12 cells. This effect is observed in both unstimulated transfected PC12 cells and transfected PC12 cells

stimulated with NGF. NGF is also known to have a transient proliferative effect on PC12 cells, which precedes the better known anti-mitogenic effects of NGF on these cells. Thus, by suppressing Ras-dependent proliferative effects, Rin2 may play a role in mediating the anti-mitogenic effects of NGF on these neuronal cells. Taken together, these data indicate that the expression of the *rin2* gene transcript can be differentially regulated in mast cells and in PC12 cells via different ligand-receptor interactions.

As disclosed herein, activation of a mouse T cell lymphoma line (EL-4 cells) via the T cell receptor, by stimulating the cells with antibodies directed against CD3 and CD28, also elicits a rapid and transient induction of expression of *rin2* mRNA transcript. T cells are essential for the expression of an adequate immune response, but inappropriate activation of T cells is thought to contribute to a wide variety of diseases with "autoimmune" components, such as autoimmune (Type I) diabetes mellitus, rheumatoid arthritis, ankylosing spondylitis, sarcoidosis, Sjögren's syndrome, multiple sclerosis, inflammatory bowel disease (i.e., Crohn's disease and ulcerative colitis), dermatomyositis, scleroderma, polymyositis, systemic lupus erythematosus, biliary cirrhosis, autoimmune thyroiditis, and autoimmune hepatitis, as well as many dermatological disorders, including psoriasis, contact sensitivity and atopic dermatitis (Schwartz, Autoimmunity and Autoimmune Diseases, in *Fundamental Immunology*, Third Edition, Paul, ed. (Raven Press, NY, pp. 1033-1097 (1993)); Rich, *Clinical Immunology*: Principles and Practice, Mosby, St. Louis (1996)). T cells are also important in the

process of tissue graft rejection, such as grafts of bone marrow, hematopoietic stem cells, skin, heart, lung, liver, kidney, pancreas and intestine, among others, and in graft-versus-host disease; T cells can also undergo neoplastic transformation, resulting in the development of leukemias or lymphomas. The findings with EL-4 T cells disclosed herein support the role of Rin2 as a negative Ras effector/regulator mediating diverse cellular responses in T cells. Taken together with the results disclosed herein regarding the role of Rin2 in regulating cellular responses in mast cells and PC12 adrenal pheochromocytoma cells, the findings in EL-4 T cells are also consistent with the hypothesis that Rin2 is a negative Ras effector/regulator mediating diverse functional responses in different cell types. There are likely to be many other cell types in which Rin2 may down-regulate Ras-dependent functional responses, since Ras is involved in influencing functional responses in diverse cell types (Marshall, *FASEB J* 9:1311-1318 (1995)). Additionally, cells which express the FcεRI receptor, T cell receptor, TrkA and c-kit, as well as diverse other receptors which signal by mechanisms which are influenced by Ras, can have their Ras-dependent functional responses negatively regulated by Rin2.

It will be apparent to the skilled artisan that the identification of the novel protein Rin2, which can inhibit Ras-dependent cellular signaling, has many commercial applications. The Ras effector signaling pathways have been shown to be important in mediating signal transduction from the cell membrane to the

nucleus in many oncogenic, mitogenic, and developmental processes.

As a result of Rin2's ability to inhibit IgE and antigen-dependent release of mediators from mast cells, compositions which can enhance Rin2 expression and/or function (e.g., Rin2 itself or other compounds), or which mimic these actions (e.g., molecules synthesized based on the structure/functional characteristics of the Rin2 protein), can be used to inhibit mediator release from effector cells in disorders such as asthma and other allergic diseases (e.g., hay fever and atopic eczema), thus ameliorating these disorders.

The ability of Rin2 to negatively regulate Ras-dependent signal transduction in other cell types (in addition to mast cells) suggests that compounds (e.g., isolated Rin2, Rin2 "mimics" or agents that can enhance expression of native Rin2) that are able to block such Ras-dependent cellular signaling pathways can produce clinically desirable effects. Mutated forms of cellular *Ras* genes, resulting in oncogenic activation of Ras proteins, have been found to be among the most common genetic abnormalities associated with human cancer. The early stage in multistage tumor progression has been associated with Ras activation, and disruption of the oncogenic *Ras* genes in colon cancer cell lines has been shown to revert the cells to a more regulated state of growth (Shirasawa *et al.*, *Science 260*:85-88). Thus, the *rin2* cDNA clone can be used to identify compounds that can block Ras-induced cell transformation *in vitro*. These compounds can include isolated Rin2 (e.g., overexpression of Rin2), derivatives (modified forms) of Rin2, and compounds identified by another

method, e.g., computer modeling of compounds designed with reference to structures and/or characteristics of Rin2 and synthesized by known methods (e.g., chemical synthesis, peptide chemistry). These compounds can be tested for their ability to function as Ras-specific anti-neoplastic agents for the treatment of cancer, or as Ras-specific agents which inhibit other examples of clinically unwanted, but non-neoplastic, Ras-dependent cellular proliferation.

Mutations in the genes encoding other molecules which are involved in Ras-dependent intracellular signaling have been linked to human diseases with a genetic component, such as some cases of melanoma. The *rin2* cDNA clone can be used to map, identify and isolate or clone the human counterpart of the *rin2* gene and, ultimately, to assess whether mutations of Rin2 and/or genes identified using Rin2 are linked to known human diseases.

The *rin2* cDNA clone can be used in a yeast two-hybrid system to identify other novel cellular proteins that interact with Rin2 and/or Ras and thus regulate the signaling responses along Ras effector pathways. Using a yeast two-hybrid system (Fields and Sternglanz, *Trends Genet. 10*:286-292 (1994)), specific regions of the Rin2 protein that interact with Ras can be identified. The elucidation of the mechanism of protein-protein interaction between Rin2 and Ras will be useful in the development of small molecule drugs that interact specifically with Ras and inhibit or enhance its function.

Using the *rin2* cDNA clone, the Rin2 protein can be produced using a protein expression system. The

partially purified Rin2 protein, or a synthetic peptide based on the known Rin2 amino acid sequence, can be used to raise Rin2-specific antibodies which can be utilized as laboratory reagents for studying the Ras effector pathways, as well as "humanized" antibodies for potential clinical applications. Rin2 is an intracellular protein. Thus, small fragments of antibodies against Rin2, perhaps specifically modified to enhance their ability to identify specific cell types and to pass through the cells' surface (plasma membrane), may be required to gain appropriate access to the intracellular Rin2 target.

However, other natural or newly-synthesized chemicals that, because of their properties, can readily enter cells and achieve intracellular concentrations which are sufficient to permit the expansion of the desired interactions with Rin2, can be identified via their ability to interact with Rin2, Rin2 fragments or Rin2-mimicking compounds, in various *in vitro* systems. Rin2-mimicking compounds are defined herein as peptide or protein compounds which may be unrelated to Rin2 in amino acid sequence, or non-peptide based compounds, which express sufficient structural similarity to native Rin2 or fragments thereof to permit such compounds to be used for initial screening of compounds, including those generated through recombinatorial chemistry approaches, to identify those which can interact with active Rin2 sufficiently to enhance or inhibit its function.

As used herein, "inhibition" is intended to encompass any reduction in the quality or quantity of the assessed activity, including complete abolishment thereof. The term "enhancement" as used herein is

intended to mean any increase in the quality or quantity of the assessed activity, including increases resulting from mimicking of an assessed activity. As used herein, "alteration"" is intended to mean either inhibition or enhancement of the quality or quantity of the assessed activity. As used herein, "mimicking" is intended to mean that the assessed activity is substantially similar to the corresponding activity of Rin2.

Depending on the circumstances, it may be desirable either to inhibit or to enhance Rin2 activity, in order to enhance or to suppress, respectively important Ras-dependent signaling pathways. Several examples of the inhibition of Rin2 activity, resulting in reduced Rin2-dependent inhibition of Ras-dependent pathways, with subsequent enhancement of such pathways, can be envisioned. Enhancement of Ras-dependent pathways promoting the proliferation and functional activation of cells involved in wound healing, including the development of new blood vessels (i.e., angiogenesis) in areas of ischemic tissue (e.g., in tissues such as cardiac or skeletal muscle, which are ischemic because of the consequences of arteriosclerosis), can have great therapeutic benefit. Enhancement of the Ras-dependent pathways which contribute to the proliferation of, and production of mediators and cytokines by, T cells and mast cells, and other cell types activated through the same or distinct receptors, may be of great benefit in diseases that are associated with diminished numbers and/or function of these cells, such as in patients infected with the human immunodeficiency virus (HIV), or receiving treatment with radiation or chemical agents for the treatment of cancer. Enhancement of Ras-

dependent signaling in neuronal cells in response to NGF or other neurotrophic factors may be desirable in settings characterized by neuronal loss, or when neural regeneration is an objective (Satoh *et al.*, *Mol. Cell. Biol.* 7:4553-4556 (1987); Szeberʹnyi *et al.*, *Mol. Cell. Biol.* *10*:5324-5332 (1990); Qiu and Green, *Neuron* 7:937-946 (1991); Muroya *et al.*, *Oncogene* 7:277-281 (1992)). For example, Ras-dependent pathways, such as the functional responses elicited by FcεRI aggregation, can be inhibited by enhancing the activity of Rin2. Agents which enhance Rin2 activity include, but are not limited to, isolated Rin2, Rin2 mimics or agents that can enhance expression of native Rin2.

In contrast, enhancement of Rin2 activity, resulting in increased Rin2-dependent inhibition of Ras-dependent pathways, with subsequent inhibition of such pathways, can also be useful. For example, it may be desirable to inhibit TCR- and Ras-dependent pathways promoting the proliferation and functional activation of cells involved in tissue graft rejection, graft-versus-host disease, and autoimmune disorders or T cell-associated disorders, e.g., autoimmune (Type I) diabetes mellitus, rheumatoid arthritis, ankylosing spondylitis, sarcoidosis, Sjögren's syndrome, multiple sclerosis, inflammatory bowel disease (i.e., Crohn's disease and ulcerative colitis), dermatomyositis, scleroderma, polymyositis, systemic lupus erythematosus, biliary cirrhosis, autoimmune thyroiditis, and autoimmune hepatitis, as well as many dermatological disorders, including psoriasis, contact sensitivity and atopic dermatitis. In asthma and allergic diseases such as hay fever and atopic dermatitis, it is desirable to inhibit

the Ras-dependent functional responses induced by FcεRI aggregation in cells bearing the FcεRI receptor, e.g., mast cells. Specifically, the intracellular signaling (e.g., activation of MAP kinase) and cellular secretory responses (e.g., release of preformed mediators, such as 5-HT, and cytokines, such as IL-6) can be inhibited by enhancing Rin2 activity. Additionally, enhancement of Rin2 activity can also be used to inhibit cellular proliferation through the TrkA receptor, e.g., in PC12 cells, and to inhibit Ras-dependent cellular proliferation, including cancers or other unwanted neoplastic or non-neoplastic proliferation of cells.

Alteration of Rin2 activity can be accomplished by increasing or decreasing the amount of Rin2 protein present to inhibit or enhance, respectively, Ras-dependent pathways, the activity of the Rin2 protein, or both. For example, the activity of the Rin2 protein described herein can be enhanced by overexpressing isolated Rin2 protein from an appropriate expression construct in a host cell. Alternatively, a cell population of interest can be treated with one or more agents, such as SCF, that enhance expression or activity of native Rin2 present in the cell population. Additionally, compounds which mimic Rin2 activity are useful in applications in which native or isolated Rin2 are useful. Useful compounds will be able to mimic at least one appropriate activity of Rin2, e.g., binding activity, catalytic activity, and include fragments and derivatives of Rin2. Alternatively, Ras-dependent pathways can be enhanced by inhibiting the activity of Rin2. Agents which inhibit Rin2 activity include, but

are not limited to, agents that can inhibit expression of native Rin2 and agents which bind native Rin2.

The invention also pertains to methods of inhibiting functional responses induced by Ras-dependent signaling pathways in a mammal by administering to the mammal an effective amount of an agent which enhances the activity of Rin2. The invention also pertains to methods of inhibiting cellular proliferation in a mammal by administering to the mammal an effective amount of an agent which enhances the activity of Rin2. The invention also encompasses methods of inhibiting Ras-dependent cellular proliferation, including cancers, neoplastic and non-neoplastic proliferation, in a mammal by administering to the mammal an effective amount of an agent which enhances the activity of Rin2.

The invention also pertains to methods of enhancing functional responses induced by Ras-dependent signaling pathways in a mammal by administering to the mammal an effective amount of an agent which inhibits the activity of Rin2.

The present invention also relates to an assay for identifying agents which alter the activity of Rin2, such as agents which enhance or inhibit the activity of Rin2. For example, a cell containing the Rin2 protein, or an active fragment or derivative thereof, can be activated via a specific receptor that induces signaling that is modulated by Rin2, in the presence of an agent to be tested, and the level of Rin2 activity can be assessed; in a preferred embodiment, the level of Rin2 activity can be assessed and compared with the corresponding level in a control in the absence of the agent to be tested. The activity of Rin2 protein can be

assessed directly, e.g., by assessing the expression levels of the protein, or indirectly, such as by assessing functional responses e.g., activation of MAP kinase activity or secretion of 5-HT or IL-6. The cell containing Rin2, or a derivative or portion thereof having Rin2 activity, can be contacted directly or indirectly with the stimulus in the presence of the agent to be tested. Enhancement of Rin2 activity, or an increase in the level of Rin2 activity relative to a control, indicates that the agent is an agonist of Rin2 activity. Similarly, inhibition of Rin2 activity, or a decrease in the level of Rin2 activity relative to a control, indicates that the agent is an antagonist of Rin2 activity.

The present invention also relates to novel agents identified by the assay described above. Agents identified by the assay described herein may enhance (e.g., prolong or increase) or inhibit (e.g., shorten or decrease) the activity of the Rin2 protein.

The following Examples are offered for the purpose of illustrating the present invention and are not to be construed to limit the scope of this invention. The teachings of all references cited herein are hereby incorporated herein by reference.

EXAMPLES

MATERIALS AND METHODS

Cytokines, cDNA Probes and Antibodies

Recombinant mouse IL-3 was purchased from BioSource International (Camarillo, CA). The c-*fos* probe is a 1.2 kb HindIII/EcoRI mouse cDNA fragment generously provided by Dr. Brent Cochran. The cDNA probes were labeled with [α-^{32}P] dCTP (6000 mCi/mmol) (New England Nuclear, Boston, MA) using the exo(-) Klenow fragment of DNA polymeRase I with random nonamer primers (Stratagene, La Jolla, CA). Purified mouse 2.5 S NGF was purchased from Upstate Biotechnology (Lake Placid, NY). For T-cell activation experiments, purified hamster anti-mouse CD3e and anti-mouse CD28 monoclonal antibodies were purchased from Pharmingen (San Diego, CA).

Cell Culture

Bone marrow-derived cultured mast cells (BMCMCs) were obtained by maintaining the femoral bone marrow cells of 4-6 week-old BALB/c mice in suspension in IL-3-containing conditioned medium, consisting of 10% heat-inactivated fetal calf serum (FCS) (Sigma Chemical Company, St. Louis, MO), 5 x 10^{-5} M 2-mercaptoethanol (Sigma) and 2 mM L-glutamine (GIBCO Laboratories, Grand Island, NY) in Dulbecco's Modified Eagle's Medium (DMEM; GIBCO Laboratories, Grand Island, NY) (complete medium) supplemented with 20% (v/v) of either supernatants from Concanavalin A-activated spleen cells or WEHI-3 cell-conditioned medium (Nabel *et al.*, *Nature* *291*:332-334 (1981); Galli *et al.*, *J. Cell Biol.* *95*:435-444 (1982)). The cells were resuspended in fresh

conditioned medium 1-2 times a week. After 4-5 weeks, at least 95% of cells that remained in the cultures were identifiable as mast cells, as determined by neutral red or May-Grünwald/Giemsa staining. C1.MC/C57.1 cells, a cloned growth factor-independent mouse mast cell line of BALB/c origin (Young *et al.*, *Proc. Natl. Acad. Sci. USA* *84*:9175-9179 (1987); Tsai *et al.*, *FASEB J. 10*, Abstract A1268 (1996)), were maintained in complete medium lacking exogenous growth factors. PC12 cells, a rat adrenal pheochromocytoma cell line, were obtained from American Type Culture Collection (ATCC No. CRL1721). They were grown in RPMI 1640 medium supplemented with 10% horse serum (HS) and 5% FCS. EL-4 cells, a mouse lymphoma T cell line, were obtained from ATCC (ATCC No. TIB39) and grown in DMEM medium supplemented with 10% HS.

FcεRI Cross-Linking

The IgE anti-DNP mAb-producing hybridoma H1-DNP-ε-26 (Liu *et al.*, *J. Immunol. 124*:2728-2737 (1980)), generously provided by Drs. F.-T. Liu and D.H. Katz, was used to generate ascites. BMCMC or C1.MC/C57.1 mast cells were sensitized with the ascites diluted 500-fold in complete medium (final IgE concentration of approximately 8 μg/ml) for 2 hours at 37°C, washed with medium, then resuspended with medium containing DNP_{30-40}-HSA (Sigma) at 50 ng/ml at 37°C. These conditions of sensitization with IgE and stimulation with specific antigen have been shown to be adequate for the activation of such cells for [^{3}H] 5-HT

release (Gordon and Galli, *J. Exp. Med.* 174:103-107 (1991)).

mRNA Differential Display

Differential display was performed using the RNAmap KitA (GenHunter, Nashville, TN) according to the manufacturer's specifications. Specifically, total RNA was extracted from cultured cells using the RNAzol B method (Biotecx Laboratories, Houston, TX) and subsequently digested with DNaseI (1 unit/10 μg) (Promega, Madison, WI) for 30 minutes at 37°C. Approximately 2 μg of digested RNA was subjected to the reverse transcription reaction with one of the four $T_{12}MN$ anchored primers (N=G, A, T, or C) at 65°C for 5 minutes, at 37°C for 60 minutes, and then at 95°C for 5 minutes. The MMLV reverse transcriptase (100 units) was added after 10 minutes of incubation at 37°C. The resulting cDNAs were further amplified by PCR (final volume, 20 μl) with GeneAmp 1XPCR Buffer (Perkin Elmer, Foster City, CA), 2 μMdNTPs, 0.2 μM AP-primers (AP-1: 5'-AGCCAGCGAA-3' (SEQ ID NO: 3); AP-2: 5'-GACCGCTTGT-3' (SEQ ID NO: 4); AP-3: 5'-AGGTGACCGT-3' (SEQ ID NO: 5); AP-4: 5'-GGTACTCCAC-3' (SEQ ID NO: 6); and AP-5: 5'-GTTGCGATCC-3' (SEQ ID NO: 7)), 1 μM $T_{12}MN$ primer (the same primer used in the respective reverse transcription reaction), 2 μl reverse transcription mix, 1 μl ^{35}S-dATP (1200 Ci/mmol) (NEN, Boston, MA), and 1 unit AmpliTaq DNA polymeRase (Perkin Elmer) at 94°C for 30 seconds, 40°C for 2 minutes, and 72°C for 30 seconds for 40 cycles, followed by an additional 5 minutes at 72°C. 3.5 μl of each sample was run on 6% sequencing gels.

Differentially-expressed PCR products were excised from the gel, extracted with water, and reamplified by PCR using the same set of primers. The reamplified PCR segments were ^{32}P-labeled as probes for Northern blot analysis, or subcloned into pGEM-T Vector (Promega) and sequenced using Sequenase version 2.0 (U.S. Biochemical, Cleveland, OH).

Northern Hybridization

Total RNA was prepared from cultured cells or from mouse tissues using the RNAzol B method (Biotecx) according to the manufacturer's specifications; 10 or 15 μg of total RNA were loaded in each lane and electrcphoresed in 1% agarose-formaldehyde denaturing gel, and then transferred onto Zetabind nylon membrane (Cuno, Meriden, CT). RNA blots were hybridized at 42°C for 16-18 hours with 10^6 cpm/ml of ^{32}P-labeled cDNA probes, washed at 42°C to a final stringency of 0.2X SSC, then exposed to Kodak XAR-5 films at -80°C.

cDNA Library Screening

The 60-4 probe isolated from the differential display studies (see Results) was radiolabeled and used as a probe to screen a BMCMC cDNA library, which had been constructed in our laboratory using the Uni-ZAP XR Vector (Stratagene, La Jolla, CA). One positive clone, SY-6, with 1.1 kb insert was isolated. To further isolate the full-length cDNA, the SY-6 clone was used as a probe to screen a mouse brain cDNA library in Uni-ZAP XR Vector purchased from Stratagene. Both libraries were screened by filter replica hybridization, and

plaque-purified phages were isolated with three rounds of screening. *In vivo* excision of plasmids from the lamda vector was performed using ExAssist helper phage and SOLR recipient cells (Strategene).

***In Vitro* Transcription/Translation**

Approximately 1 μg of the pBluescript SK (-) containing the full-length SY-A cDNA clone was transcribed and translated *in vitro* using the TNT T_3 Coupled Reticulocyte Lysate System (Promega). Proteins were labeled in the translation step with 40 μCi of [^{35}S] methionine (1,000 Ci/mmol) (Amersham, Arlington, IL), run on an 8% SDS/PAGE, and visualized by autoradiography.

Rin2 Antibody and Western Blotting

Anti-Rin2 antibodies were prepared by immunizing rabbits with a synthetic peptide corresponding to the 16 N-terminal residues of murine Rin2 (KSERRGIHVDQSELLC; SEQ ID NO: 16) conjugated to keyhole limpet hemacyanin. The antibody was affinity-purified on a peptide column using a cysteine residue of the peptide coupled to an iodoacetamide on Sepharose beads. Cells were lysed in lysis buffer (20 mM Tris 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerolphosphate, 1 mM Na_3VO_4, 1 μg/ml leupeptin, 1 mM PMSF). Resulting cell lysates were subjected to 12% SDS-PAGE and electroblotted. Membranes were incubated with the anti-Rin2 antibody (1:500), and the antigen-antibody complexes were visualized with anti-rabbit IgG

secondary antibody using enhanced chemiluminescence (ECL).

Yeast Two-Hybrid Assay

The protein-protein interactions between murine Rin2 and the murine or yeast Ras protein were assessed using the MATCHMAKER Two-Hybrid System1 (Clontech) according to the instructions supplied by the manufacturer. The entire open reading frame, or segments of the open reading frame of the SY-A clone was subcloned, in frame, into the pACT2 vector. The murine H-Ras cDNA was generated from total RNA of C1.MC/C57.1 by RT-PCR using the 5' primer GCGGAATTCATGACAGAATACAAGCTTGTG (SEQ ID NO: 17) and the 3' primer GACGGATCCCTCAGGACAGCACACACTTGC (SEQ ID NO: 18) and subcloned into pAS2-1. The yeast Ras2p cDNA was generated from *S. cerevisiae* poly A+ RNA (Clontech) by RT-PCR using the 5' primer CGATGTCGACCATGCCTTTGAACAAGTCG (SEQ ID NO: 19) and the 3' primer GATAGGATCCACCCGATCCGCTCTTG (SEQ ID NO: 20) and subcloned into pAS2-1. A Cys-to-Ser mutation at residues 318 of Ras2p, which suppresses palmitolylation, was constructed using the QuikChange Site-Directed Mutagenesis Kit (Stratagene). Y187 yeast strain was cotransformed with the constructs described above and the transformants were selected on -Leu/-Trp plates. β-galactosidase activity was determined for colonies that appeared after 3-4 days by a qualitative colony-lift filter assay using X-gal as substrate and a quantitative liquid culture assay using CPRG as substrate.

Generation of Stable Transfectants

The SY-A cDNA clone was digested with XbaI and XhoI restriction enzymes, and the entire full-length cDNA insert released was ligated to the pBK-CMV expression vector (Stratagene) to generate the *rin2* antisense expression plasmid, pBK-CMV-SYAS. Plasmid pBK-CMV-SYAS and the vector control plasmid pBK-CMV were transfected into C1.MC/C57.1 mast cells or PC12 cells using LIPOFECTAMINE Reagent (Life Technologies, Gaithersburg, MD) according to the manufacturer's instructions. For C1.MC/C57.1 cells, transfected cells were selected in Geneticin (G418) (Life Technologies) at 2 mg/ml for 10 days and then at 0.8 mg/ml thereafter. G418-resistant mast cell colonies were observed approximately two weeks after transfection. For PC12 cells, transfected cells were selected in 0.6 mg/ml G418 and G418-resistant colonies were observed six weeks after transfection.

Measurement of Mediator Release

The release of 5-HT from mast cells was measured as previously described (Coleman *et al.*, *J. Immunol.* *150*:556-562 (1993)). Specifically, ^{3}H-hydroxytryptamine creatinine sulfate (25.2 Ci/ mmol) (NEN) was added to mast cells 2 hours before stimulation. The cells were washed to remove unincorporated ^{3}H-5-HT and stimulated with IgE and specific antigen as described above. Release of radioactivity in supernatant fractions (150 μl) was determined after stimulation, and net percentage release was calculated as [(a-b)÷c] X 100, where "a" is radioactivity released by stimulated cells, "b" is radioactivity released by unstimulated cells, and "c" is

total incorporated cellular radioactivity that was measured after cells were lysed in 0.05% Triton X-100 (Sigma). IL-6 release was assayed using enzyme-linked immunosorbent assay ELISA kits (ENDOGEN, Boston, MA) according to the manufacturer's instructions. Release of β-hexosaminidase from transfectants was measured on an ELISA reader using *p*-nitrophenyl-N-acetyl-β-D-glucosamine (Sigma) as the substrate as described by Berger in Immunology Methods Manual Vol. 3 (ed. Lefkovits, I.):1436-1440 (Academic Press, San Diego, 1997).

Kinase Assays

The p44/p42 Erk-MAP kinase activity was assayed using the MAP Kinase Assay Kit (New England Biolabs, Beverly, MA). Specifically, cells were lysed by sonication in 0.5 ml of cell lysis buffer (20 mM Tris 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerolphosphate, 1 mM Na_3VO_4 1 μg/ml leupeptin, 1 mM PMSF). 0.2 ml of cell lysate was then incubated with the phospho-specific p44/p42MAP kinase antibody (1:100 dilution) overnight at 4°C and subsequently with Protein A/G PLUS-Agarose (Santa Cruz Biotechnology, Sant Cruz, CA) for 3 hours at 4°C. Immunoprecipitates were incubated in 50 μl 1X kinase buffer (25 mM Tris 7.5, 5 mM β-glycerolphosphate, 2 mM DTT, 0.1 mM Na_3VO_4, 10 mM $MgCl_2$) supplemented with 100 mM ATP and 1 μg of Elk1 fusion protein substrate for 30 minutes at 30°C. The reaction mixture was subjected to 12% SDS-PAGE gel electrophoresis, and then electroblotted onto Immobilon-P transfer membrane

(Millipore, Bedford, MA). Membranes were incubated with the phospho-specific Elk1 (Ser383) antibody (1:1000 dilution), and the antibody was visualized with horseradish peroxidase-conjugated anti-rabbit IgG secondary antibody using the Phototope-HRP Western Detection Kit (New England Biolabs, Beverly, MA). The films were subsequently scanned using a densitometer (Molecular Dynamics, Sunnyvale, CA) and specific signals for phospho-Elk1 were quantitated by ImageQuant software (Molecular Dynamics).

The activation of JNK activity was assayed using the SAPK/JNK Assay Kit (New England BioLabs). Specifically, total JNK was immunoprecipitated using an N-terminal c-Jun (1-89) fusion protein bound to glutathione sepharose beads and incubated with ATP in the presence of kinase buffer. The phosphorylation of c-Jun by JNK was measured by Western blotting using a phospho-specific c-Jun antibody.

The activation of p38 MAP kinase activity was assayed using the p38 MAP Kinase Assay Kit (New England BioLabs). Specifically, total p38 MAP kinase was immunoprecipitated using a p38 MAP kinase antibody and incubated with GST-ATF-2 fusion protein in the presence of ATP and kinase buffer. Phosphorylation of ATF-2 was measured by Western blotting using a phospho-specific ATF-2 antibody and ECL.

Statistical Analysis

The results for differences in mediator release were analyzed for statistical significance by the

unpaired Student's *t* test (two-tailed). All results are expressed as the arithmetic mean ± SEM.

RESULTS

Identification of a Differentially-Expressed mRNA in Mouse Mast Cells Stimulated with IgE and Antigen

To isolate genes involved in FcεRI-mediated signaling, the C1.MC/C57.1 cell line, a cloned growth factor-independent mouse mast cell line of BALB/c origin (Young *et al.*, *Proc. Natl. Acad. Sci. USA 84*:9175-9179 (1987); Tsai *et al.*, *FASEB J. 10*, Abstract A1268 (1996)), was used. It has been reported that C1.MC/C57.1 cells can exhibit many functional cellular responses characteristic of activated mast cells, including the release of pre-formed mediators and cytokines upon FcεRI receptor crosslinking (Gordon and Galli, *J. Exp. Med. 174*:103-107 (1991)). C1.MC/C57.1 mast cells were sensitized with anti-DNP IgE mAb and challenged with $DNP_{30-40}HSA$ or vehicle alone for 30 minutes, 1 hour, or 2 hours. Total RNAs were extracted from the stimulated or unstimulated mast cells, and subjected to mRNA differential display analysis as described (Liang and Pardee, *Science 257*:967-971 (1992); Liang *et al.*, *Nucl. Acids Res. 21*:3269-3275 (1993)).

Twenty-six differential display experiments were performed using different combinations of 3' anchored primers and 5' arbitrary primers and different RNA isolates from various time intervals of stimulation. Total RNA was extracted from C1.MC/C57 mast cells that had been activated by IgE and specific antigen for 1 hour, and then amplified using $T_{12}MT$ anchored primers and

the AP-5 arbitrary primer (5'-GTTGCGATCC) (SEQ ID NO: 7). Several differentially expressed bands were consistently detected, including band 60-4.

Band 60-4 was excised from the gel, reamplified, and used as probe for subsequent Northern blot analysis. The 60-4 cDNA probe hybridized to a 2.8 kb mRNA whose expression was rapidly increased in C1.MC/C57.1 mouse mast cells that had been activated through the FcεRI for 30 minutes or 1 hour, but returned to baseline levels after 2 hours. The 60-4 probe was subsequently subcloned, and sequence analysis revealed a 328 base pair cDNA with no significant homology to any other entry in the Genbank database.

Cloning and Structure of Murine Rin2

The 60-4 probe was used to screen a BALB/c mouse bone marrow-derived cultured mast cell (BMCMC) cDNA library constructed in the Uni-ZAP XR vector (Stratagene). One positive clone (SY-6) with a 1.1 kb insert was isolated. Sequencing of the entire SY-6 partial cDNA clone revealed no significant homology with any known genes. To facilitate the cloning of the full-length cDNA, the SY-6 clone was used as a probe to assess the expression pattern of the identified novel gene transcript by Northern blot analysis. The expression of this novel mRNA appeared widespread, as it was detected in many tissues, including the heart, liver, kidney, lung, skeletal muscle, testis spleen and brain. Because the identified gene transcript was expressed fairly abundantly in the brain, the SY-6 partial cDNA clone was used to screen a mouse brain cDNA

library (Stratagene) in an attempt to obtain the full-length cDNA clone. Three positive clones (SY-A, SY-B, SY-C) were identified after the tertiary screening, and clone SY-A appeared to contain an insert (2.6 kb) of the appropriate predicted size (2.8 kb).

Sequencing of the entire SY-A clone (2664 base pairs) revealed a potential translational initiation codon (ATG) at base 60 and a stop codon (TGA) at base 1534. The predicted open reading frame (ORF) of 1476 base pairs encodes a predicted protein of 491 amino acids with a molecular mass of 56.9 kDa (Figure 3). To determine whether the SY-A cDNA could indeed express a protein of the predicted size, the SY-A clone was subcloned into a pGEM expression vector and subjected to coupled *in vitro* transcription/translation in a wheat germ lysate system. A specific product of an apparent molecular mass of 60 kDa was detected. These results are thus consistent with the size of the predicted protein deduced from the predicted ORF. Moreover, hydrophilicity plot (Kyte-Doolittle) analysis of the predicted ORF revealed a highly hydrophilic protein with a few short hydrophobic regions. Thus, the protein product of the predicted ORF is likely to be a soluble cytosolic protein.

The predicted amino acid sequence of SY-A cDNA was used to search available protein databases using the BLAST program network server. Over a particular stretch of amino acid sequence, the SY-A gene product was found to share significant sequence homology with members of a putative GTPase-binding protein family such as Vps9p (BLAST *P*= 2.2e-19), JC265 (*P*= 5.2e-05), and Rin1 (*P*= 4.1e-05) (Figure 4). Vps9p is a recently characterized

yeast protein required for vacuolar protein sorting (Burd *et al.*, *Mol. Cell. Biol. 16*:2369-2377 (1996)). The two mammalian proteins encoded by two closely related human cDNAs, JC265 and Rin1, have been identified as inhibitors of an activated Ras2 allele in *S. cerevisiae* (Colicelli *et al.*, *Proc. Natl. Acad. Sci. USA 88*:2913-2917 (1991); Han and Colicelli, *Mol. Cell. Biol. 15*:1318-1323 (1995)). Alignment of the amino acid sequences of these proteins with that of SY-A showed that the highest homology was found in the GTPase binding homology (GBH) domain regions in which three GBH motifs have been described (Burd *et al.*, *Mol. Cell. Biol. 16*:2369-2377 (1996)). Specifically, the SY-A protein contains a 148-amino acid sequence which is 34% identical (51/148) and 22% similar (33/148) to the respective GBH domains of Vps9p containing the three GBH motifs (Figure 4). Moreover, the SY-A gene product contains an amino acid sequence (SADDFLPTL; SEQ ID NO: 8) that matches well with the proposed GBH motif II (GADXFLPVL; SEQ ID NO: 9), and another downstream sequence (GEDGYYFTN; SEQ ID NO: 10) that also aligns well with the proposed GBH motif III (GEXXYYLTS; SEQ ID NO: 11) (Burd *et al.*, *Mol. Cell. Biol. 16*:2369-2377 (1996)) (Figure 4). Interestingly, the GBH motif II is situated in a region of JC265 and Rin1 that has been previously suggested to be distantly related to several Ras-interacting proteins such as the human GAP, the yeast IRA1 and IRA2 proteins, NF1, and sar1 (Colicelli *et al.*, *Proc. Natl. Acad. Sci. USA 88*:2913-2917 (1991)). Because of its significant homology to the mammalian protein Rin1, the protein encoded by the SY-A cDNA is

herein called Rin2 (also called Rabex-5), and the gene encoding the protein is herein called *rin2*.

Interation of Rin2 with Ras

Since Rin1 can suppress an activated Ras allele in yeast and interact directly with yeast Ras2p and with wild-type and activated human H-Ras (Han and Colicelli, *Mol. Cell. Biol. 15*:1318-1323 (1995); Han, *Proc. Natl. Acad. Sci. USA 94*:4954-4959 (1997)), it was of interest to determine whether Rin2 could also interact directly with Ras. Using the yeast two-hybrid system with the entire open reading frame (residues 1-491) of murine Rin2 expressed as a GAL4-activation domain fusion protein and the wild-type murine H-Ras expressed as the DNA-binding fusion protein, it was demonstrated that murine Rin2 was able to interact with wild-type murine H-Ras (Figure 12). Positive interactions with murine H-Ras could also be detected for the N-terminal and the C-terminal regions of Rin2, and for the region of Rin2 containing the three GBH domains (residues 203-380). But the strength of interaction with murine H-Ras protein, as measured by a quantitative β-galactosidase assay, appeared to be the strongest for the full-length murine Rin2 protein (Figure 12). However, unlike Rin1, full length murine Rin2 proteins did not appear to bind significantly to the wild-type yeast Ras2p, nor did any of the three truncated fusion protein constructs representing the three different regions of the Rin2 protein. These data indicate that Rin2 can interact with Ras in ways that are distinct from those of Rin1.

Decreased Expression of Rin2 Enhances Signaling Responses Induced by FcεRI Aggregation in Mast Cells

The FcεRI-induced activation of Ras in mast cells results in an increase in transcriptional activity of the transcription factor Elk-1 (Turner and Cantrell, *J. Exp. Med. 185*:43-53 (1997)), which is a regulator of the serum response element in the promoter region of immediate early response genes such as c-*fos* (Treisman, *Curr. Opin. Genet. Dev.* 4:96-101 (1994)). The transcription activation of Elk-1 is mediated by the Ras/Raf-1/MEK/Erk-MAP kinases pathway (Treisman, *Curr. Opin. Cell Biol. 8*:205-215 (1996)). As described herein, *rin2* mRNA was identified by virtue of its enhanced expression in mast cells that are activated by FcεRI aggregation. Because Rin1 has been indicated as a negative effector of Ras, the ability of Rin2 to suppress the signaling responses initiated by Ras in mouse mast cells activated by FcεRI ligation was assessed.

The SY-A cDNA was inserted into a CMV expression vector (pBK-CMV) in the antisense orientation, and the resulting plasmid (pBK-CMV-SYA-AS) was stably transfected into mouse C1.MC/C57.1 mast cells. From two independent transfections, eight separate stable lines of antisense Rin2 transfectants and seven separate stable lines of control CMV vector transfectants were established.

The G418-resistant colonies obtained were stimulated with IgE and antigen, and the pattern of expression of early response genes such as c-*fos* was assessed using Northern blot analysis. Transfection of

antisense *rin2* significantly enhanced the expression of mRNA of c-*fos* in mouse mast cells activated by FcεRI crosslinking. In mouse mast cells transfected with the control CMV plasmid, c-*fos* mRNA expression was rapidly induced by 15 or 30 minutes after IgE and antigen-dependent stimulation, but returned to undetectable or basal levels by 1 hour after stimulation. In contrast, in mouse mast cells transfected with the *rin2* antisense construct, not only was a significantly higher level of c-*fos* expression observed at 15 or 30 minutes after IgE and antigen-dependent stimulation, but a significant and sustained induction of c-*fos* message was still detectable in these activated mast cells at 1 hour after stimulation.

Similarly, the activation of Erk-MAP kinase activity induced by FcεRI activation was also greatly potentiated in C1.MC/C57.1 mast cells that had been stably transfected with the antisense *rin2* construct (Figure 5). Consistent with results reported previously (Tsai et al., *Eur. J. Immunol. 23*:3286-3291 (1993), FcεRI crosslinking caused a rapid activation of Erk-MAP kinase activity in mouse mast cells which had been transfected with the control plasmid 30 minutes after stimulation (2.2 fold increase). The activation persisted for 2 hours (approximately 1.5 fold increase) and then declined to basal levels by 3 hours after stimulation (1.0 fold). In contrast, in mouse mast cells transfected with the *rin2* antisense plasmid, a significantly higher level of Erk-MAP kinase activation was induced by FcεRI-dependent stimulation for 30 minutes (4.0 fold), 1 hour (3.6 fold), or 2 hours (2.2

fold) after the addition of antigen, and such Erk-MAP kinase activation was still sustained at 3 hours (1.3 fold).

Stimulation of mast cells by FcεRI aggregation leads to the activation of the Ras-mediated ERK-MAP kinase, JNK, and p38 signaling pathways (Tsai *et al.*, *Eur. J. Immunol.* *23*:3286-3291 (1993); Hirasawa *et al.*, *J. Biol. Chem.* *270*:10960-10967 (1995); Zhang *et al.*, *J. Biol. Chem.* *272*:13397-13402 (1997); Ishizuka *et al.*, *Proc. Natl. Acad. Sci. USA* *94*:6358-6363 (1997); Kawakami *et al.*, *J. Immunol.* *161*:1795-1802 (1998)), and activation of the ERK-MAP kinases and JNK in turn regulate the synthesis and release of cytokines from these cells. FcεRI crosslinking caused a rapid activation of ERK-MAP kinase activity in control transfected C1.MC/C57.1 cells by 30 minutes to 2 hours after stimulation, which declined to basal levels by 3 hours after stimulation. In contrast, in the Rin2 antisense transfected mast cells, a significantly higher level of ERK-MAP kinase activation was induced by FcεRI stimulation 30 minutes to 2 hours after antigen challenge, and high levels of ERK-MAP kinase activation were still sustained at 3 hours. Antisense Rin2 expression also significantly potentiated the levels of activation of JNK (Figure 14) and p38 MAP kinase (Figure 15) induced by FcεRI aggregation at 30 minutes to 3 hours after stimulation. Taken together, the results of these studies indicate that reduced expression of Rin2 can result in marked potentiation of the Ras-mediated signaling responses in mast cells, and suggest that Rin2 functions as a Ras inhibitor/effector.

Decreased Expression of Rin2 Potentiates Release of Preformed Mediator from Mast Cells Activated by FcεRI Aggregation

Since reduced expression of Rin2 can potentiate some of the intracellular signaling responses initiated by FcεRI-dependent mast cell activation, the effect of decreased Rin2 expression on the cells' secretory responses to FcεRI-dependent activation was also assessed. C1.MC/C57.1 mast cells which had been transfected with the *rin2* antisense expression or control vectors were stimulated with IgE and $DNP_{30-40}HSA$. As shown in Figure 6, mouse mast cells stably transfected with the *rin2* antisense construct released significantly higher levels of the preformed mediator, 5-HT, than did mast cells transfected with the pBK-CMV vector only. This effect was observed at all concentrations of DNP antigen tested (1, 10, 50, or 100 ng/ml $DNP_{30-40}HSA$), indicating that the potentiating effect of *rin2* antisense expression was not antigen dose-dependent.

Decreased Expression of Rin2 Potentiates Cytokine Release from Mast Cells Activated by FcεRI Aggregation

Crosslinking of FcεRI receptors not only induces mast cells to release preformed mediators such as 5-HT, but also induces these cells to secrete cytokines (Gordon *et al.*, *Immunol. Today* 11:458-464 (1990); Paul *et al.*, *Adv. Immunol.* 53:1-29 (1993)). Thus, the effect of changes in Rin2 expression on the FcεRI-dependent release of IL-6 from mouse mast cells was also assessed. As illustrated in Figure 7A, the release of IL-6 from

mast cells 2, 4, or 6 hours after FcεRI aggregation (50 ng/ml) was enhanced dramatically by the transfection of the cells with the *rin2* antisense expression vector, as compared with responses elicited in mast cells transfected with the control CMV vector. This potentiating effect was more pronounced (greater than 20-fold increase) in cells which had been stimulated for longer time intervals (4-6 hours). Furthermore, when the transfected cells were stimulated by different concentrations of $DNP_{30-40}HSA$ for 6 hours, mouse mast cells which had been transfected with the *rin2* antisense construct released significantly higher levels of IL-6 than did mast cells which had been transfected with the control vector at each concentration of antigen tested (Figure 7B).

As illustrated in Figure 13A, there were no significant differences between the amounts of β-hexosaminidase that were released from the antisense or control transfectants in response to FcεRI aggregation. This result is consistent with findings that the release of pre-formed mediators can occur independently of signaling pathways involving Ras/MAP kinase (Hirasawa *et al.*, *J. Biol. Chem.* *270*:10960-10967 (1995); Zhang *et al.*, *J. Biol. Chem.* *272*:13397-13402 (1997)). On the other hand, release of the cytokine IL-6 from the mast cell transfectants 6 hours after FcεRI aggregation was significantly enhanced (by a mean value of approximately 70%) in the antisense group (Figure 13A). The time course of this effect is shown in Figure 13B. In general, those antisense transfectants which exhibited the largest and most sustained reduction in Rin2 protein

expression after FcεRI aggregation also exhibited the greatest enhancement of IL-6 production (Figure 13C).

Rin2 mRNA Expression is Up-regulated in Mouse Mast Cells Stimulated with Stem Cell Factor and in PC12 Cells Stimulated with Nerve Growth Factor

To assess whether the enhanced expression of the *rin2* transcript was specific for the FcεRI-dependent signaling pathway in mast cells, Northern analysis was performed to examine the expression of *rin2* mRNA in mouse bone marrow derived cultured mast cells (BMCMC) which had been stimulated with the growth factor, stem cell factor (SCF), the ligand for the c-kit receptor tyrosine kinase (Galli *et al.*, *Adv. Immunol.* 55:1-96 (1994)). The widespread tissue distribution of *rin2* mRNA suggests that Rin2 may function as a signaling element in the functional responses of many cell types besides mast cells. Therefore, *rin2* expression was also analyzed in PC12 cells, an adrenal pheochromocytoma cell line, which had been stimulated with nerve growth factor (NGF) (Greene and Tischler, *Proc. Natl. Acad. Sci. USA* 73:2424-2428 (1976)).

rin2 mRNA expression was rapidly increased by 30 minutes to 1 hour after stimulation of BMCMCs with SCF (50 ng/ml), but returned nearly to baseline levels after 2 hours. Expression of the *rin2* transcript was also significantly induced 30 minutes after NGF stimulation (50 ng/ml) in PC12 cells, and the enhanced expression of the transcript was still detectable 2 hours after stimulation. Thus, SCF induced a transient enhancement of *rin2* mRNA expression in mast cells, but NGF elicited

a much more sustained activation of *rin2* expression in PC12 adrenal pheochromocytoma cells.

Decreased Expression of *Rin2* Enhances Cell Proliferation in PC12 Cells

PC12 cells stimulated with NGF undergo neuronal differentiation, which is characterized by the extension of neurites and the cessation of cell proliferation (Greene and Tischler, *Proc. Natl. Acad. Sci. USA* 73:2424-2428 (1976)). Since NGF promoted a sustained induction of *rin2* mRNA expression in PC12 cells, the effects of reduced *rin2* expression on cell proliferation rate and NGF-induced neurite outgrowth were examined in these cells. The antisense expression vector pBK-CMV-SYA-AS was stably transfected into PC12 cells, and the transfected cells were plated onto collagen-coated plates and stimulated with NGF at 25, 50 or 100 ng/ml. After 3-5 days, neurite outgrowth was observed both in PC12 cells that had been transfected with the antisense expression vector and in those cells that had been transfected with the control CMV vector. No detectable differences in the time of onset or the number of neurite-bearing cells could be observed between these populations of transfected cells. These results thus indicate that Rin2 may not be directly involved with NGF-induced neuronal differentiation in PC12 cells. On the other hand, PC12 cells which had been transfected with the antisense expression vector and then maintained in their normal culture medium exhibited a significantly higher rate of cell proliferation, as measured by a ^{3}H-thymidine assay, than did the cells which had been

transfected with the control pBK-CMV vector (Figure 8A). This effect was also observed when the transfected PC12 cells were stimulated with NGF (100 ng/ml) for 18 hours in medium containing 0.5% FCS (Figure 8B). Since NGF is known to be anti-mitogenic to PC12 cells (Greene and Tischler, *Proc. Natl. Acad. Sci. USA* 73:2424-2428 (1976)), these results are consistent with the hypothesis that Rin2 expression results in the inhibition of cell proliferation in PC12 cells and perhaps other neuronal cells.

Rin2 mRNA Expression is Up-Regulated in EL-4 T Cells Stimulated via the T Cell Receptor Complex

The binding of peptide antigen and major histocompatibility complex molecules to the T cell receptor (TCR) induces T cell activation, which includes the secretion of effector cytokines by the T cells and the induction of mitotic activity in the T cells themselves. Extensive evidence has shown that the activation of Ras is involved in some of the intracellular signaling pathways mediating T cell activation (Lowy and Willumsen, *Ann. Rev. Biochem.* *62*:851-891 (1993)). To assess whether T cell activation could result in enhanced expression of the *rin2* transcript, Northern analysis was performed to examine the expression of *rin2* mRNA in EL-4 mouse T cells that had been stimulated via the TCR by incubating the cells with both anti-CD3 antibody (1 μg/ml) and anti-CD28 antibody (2 μg/ml). *rin2* mRNA expression was rapidly increased by 30 minutes to 1 hour after TCR stimulation, but declined nearly to baseline levels after 2 hours.

The time course of this effect on *rin2* mRNA expression in T cells is thus similar to that observed in mouse mast cells stimulated with SCF or IgE and antigen.

EQUIVALENTS

Those skilled in the art will recognize, or be able to ascertain, using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims:

CLAIMS

We claim:

1. An isolated Rin2 polypeptide which down-regulates functional responses elicited by Ras-dependent signaling pathways, or a derivative or portion thereof having Rin2 activity.

2. An isolated Rin2 polypeptide according to Claim 1, wherein the Ras-dependent signaling pathway is FcεRI aggregation.

3. An isolated Rin2 polypeptide according to Claim 1, wherein said Rin2 polypeptide comprises the amino acid sequence of SEQ ID NO: 2.

4. An isolated Rin2 polypeptide according to Claim 1, wherein said Rin2 polypeptide has the same amino acid sequence as endogenous Rin2.

5. An isolated Rin2 polypeptide according to Claim 1, wherein said Rin2 polypeptide comprises an amino acid sequence which is at least about 40% similar to the amino acid sequence of SEQ ID NO: 2.

6. An isolated nucleic acid molecule which encodes a Rin2 polypeptide which down-regulates functional responses elicited by Ras-dependent signaling pathways, or a derivative or portion thereof having Rin2 activity.

7. An isolated nucleic acid molecule according to Claim 6, wherein said nucleic acid molecule comprises the nucleotide sequence of SEQ ID NO: 1 or the complement of SEQ ID NO: 1, or a derivative or portion thereof.

8. An isolated polypeptide encoded by a nucleic acid molecule according to Claim 7.

9. An isolated nucleic acid molecule according to Claim 6, wherein said nucleic acid molecule comprises one or more nucleotide sequences selected from the group consisting of:

a) SEQ ID NO: 12 or the complement of ID NO: 12;

b) SEQ ID NO: 13 or the complement of SEQ ID NO: 13;

c) SEQ ID NO: 14 or the complement of SEQ ID NO: 14;

d) SEQ ID NO: 15 or the complement of SEQ ID NO: 15; and

e) derivatives or portions of (a)-(d).

10. An isolated protein encoded by an isolated nucleic acid molecule according to Claim 8.

11. An isolated nucleic acid molecule according to Claim 6 comprising a nucleotide sequence which selectively hybridizes to all or a portion of SEQ ID NO: 1.

12. An isolated nucleic acid molecule according to Claim 6 comprising a nucleotide sequence which

selectively hybridizes to all or a portion of one or more nucleotide sequences selected from the group consisting of SEQ ID NO: 12, SEQ ID NO: 13, SEQ ID NO: 14 and SEQ ID NO: 15.

13. An isolated nucleic acid molecule according to Claim 6 comprising a nucleotide sequence which is at least about 75% identical to SEQ ID NO: 1.

14. An isolated nucleic acid molecule according to Claim 13 comprising a nucleotide sequence which is at least about 90% identical to SEQ ID NO: 1.

15. An isolated nucleic acid molecule according to Claim 6 comprising a nucleotide sequence which is at least about 75% identical to one or more nucleotide sequences selected from the group consisting of SEQ ID NO: 12, SEQ ID NO: 13, SEQ ID NO: 14 and SEQ ID NO: 15.

16. An isolated nucleic acid molecule according to Claim 15 comprising a nucleotide sequence which is at least about 90% identical to one or more nucleotide sequences selected from the group consisting of SEQ ID NO: 12, SEQ ID NO: 13, SEQ ID NO: 14 and SEQ ID NO: 15.

17. A DNA construct comprising an isolated nucleic acid molecule according to Claim 6 operatively linked to a regulatory sequence.

18. A recombinant host cell comprising an isolated nucleic acid molecule according to Claim 6 operatively linked to a regulatory sequence.

19. A method for preparing a Rin2 polypeptide, comprising culturing the recombinant host cell of Claim 18.

20. An antibody, or an antigen-binding fragment thereof, which selectively binds to the Rin2 polypeptide according to Claim 1, or a derivative or portion thereof having Rin2 activity.

21. A method for assaying the presence of a Rin2 polypeptide according to Claim 1 in a cell or cell lysate, comprising contacting said cell or cell lysate with an antibody which specifically binds to the Rin2 polypeptide.

22. A method of identifying an agent which alters activity of a Rin2 polypeptide according to Claim 1, comprising the steps of:

(a) contacting a cell containing the Rin2 polypeptide, or a derivative or portion thereof having Rin2 activity, with a stimulus which activates at least one Ras-dependent pathway in said cell, in the presence of the agent to be tested; and

(b) identifying alteration of Rin2 activity.

23. A method according to Claim 22, wherein the stimulus is selected from the group consisting of

nerve growth factor, stem cell factor, peptide antigen and major histocompatibility complex molecules, and IgE and specific antigen.

24. A novel agent which alters Rin2 activity identified according to the method of Claim 23.

25. A method of enhancing activity of a Rin2 polypeptide according to Claim 1, comprising contacting said Rin2 polypeptide, or a cell containing said Rin2 polypeptide, with an agent that enhances the activity of Rin2.

26. A method of inhibiting a functional response induced by a Ras-dependent signaling pathway in a cell, comprising contacting said cell with an agent which enhances the activity of a Rin2 polypeptide.

27. A method according to Claim 26, wherein the cell expresses a receptor having a signaling pathway regulated by Rin2.

28. A method according to Claim 27, wherein the receptor is selected from the group consisting of FcεRI, TrkA, c-kit and T cell receptor.

29. A method according to Claim 26, wherein the functional response is one or more responses selected from the group consisting of activation of ERK-MAP kinase, activation of JNK kinase and activation of p38 MAP kinase.

30. A method according to Claim 26, wherein the functional response is a cellular secretory response.

31. A method according to Claim 30, wherein the cellular secretory response is release of preformed mediators, lipid mediators and/or cytokines.

32. A method of inhibiting a Ras-dependent signaling pathway in a cell, comprising contacting said cell with an agent which enhances the activity of a Rin2 polypeptide.

33. A method of inhibiting cellular proliferation in a cell, comprising contacting said cell with an agent which enhances the activity of a Rin2 polypeptide.

34. A method according to Claim 33, wherein the cell expresses the TrkA receptor for nerve growth factor.

35. A method of inhibiting Ras-dependent signaling in a mammal comprising administering to the mammal an effective amount of an agent which enhances the activity of Rin2.

36. A method of inhibiting a functional response induced by a Ras-dependent signaling pathway in a mammal, comprising administering to the mammal an effective amount of an agent which enhances the activity of Rin2.

37. A method of inhibiting Ras-dependent signaling in the mast cells of a mammal, comprising administering to the mammal an effective amount of an agent which enhances the activity of Rin2.

38. A method of inhibiting Ras-dependent signaling in T cells of a mammal, comprising administering to the mammal an effective amount of an agent which enhances the activity of Rin2.

39. A method of inhibiting Ras-dependent cell proliferation in a mammal, comprising administering to the mammal an effective amount of an agent which enhances the activity of Rin2.

40. A method of treating an allergic condition in a mammal comprising administering to the mammal an effective amount of an agent which enhances the activity of the Rin2 polypeptide.

41. A method according to Claim 40, wherein the allergic condition is asthma, hay fever or atopic eczema.

42. A method of treating a disorder selected from the group consisting of Ras-dependent cancer, Ras-dependent neoplastic cellular proliferation and Ras-dependent unwanted non-neoplastic cellular proliferation in a mammal, comprising administering to the mammal an effective amount of an agent which enhances the activity of a Rin2 polypeptide.

43. A method of inhibiting functional responses induced by T cell receptor-dependent activation of T cells in a mammal comprising administering to the mammal an effective amount of an agent which enhances the activity of Rin2.

44. A method of treating a disorder selected from the group consisting of autoimmune disorder, T cell-associated disorder, T cell-dependent graft rejection and graft-versus-host disease in a mammal, comprising administering to the mammal an effective amount of an agent which enhances the activity of a Rin2 polypeptide.

45. A method according to Claim 44, wherein the disorder is selected from the group consisting of autoimmune (Type I) diabetes mellitus, rheumatoid arthritis, ankylosing spondylitis, sarcoidosis, Sjögren's syndrome, multiple sclerosis, inflammatory bowel disease, Crohn's disease, ulcerative colitis, dermatomyositis, scleroderma, polymyositis, systemic lupus erythematosus, biliary cirrhosis, autoimmune thyroiditis, autoimmune hepatitis, psoriasis, contact sensitivity and atopic dermatitis.

46. A method of inhibiting activity of a Rin2 polypeptide according to Claim 1, comprising contacting said Rin2 polypeptide, or a cell containing said Rin2 polypeptide, with an agent that inhibits the activity of Rin2.

47. A method of enhancing a functional response induced by a Ras-dependent signaling pathway in a cell, comprising contacting said cell with an agent which inhibits the activity of a Rin2 polypeptide.

48. A method according to Claim 47, wherein the cell expresses a receptor having a signaling pathway regulated by Rin2.

49. A method according to Claim 48, wherein the receptor is selected from the group consisting of FcεRI, TrkA, c-kit and T cell receptor.

50. A method of enhancing a Ras-dependent signaling pathway in a cell, comprising contacting said cell with an agent which inhibits the activity of a Rin2 polypeptide.

51. A method of enhancing a functional response induced by a Ras-dependent signaling pathway in a mammal, comprising administering to the mammal an effective amount of an agent which inhibits the activity of Rin2.

52. A method of enhancing Ras-dependent signaling in a mammal comprising administering to the mammal an effective amount of an agent which inhibits the activity of Rin2.

53. A method of enhancing functional responses induced by T cell receptor-dependent activation of T cells in a mammal comprising administering to the mammal

an effective amount of an agent which inhibits the activity of Rin2.

54. A method of enhancing Ras-dependent signaling in the mast cells of a mammal, wherein the desired outcome is increased mast cell function, comprising administering to the mammal an effective amount of an agent which inhibits the activity of Rin2.

55. A method of enhancing Ras-dependent signaling in T cells of a mammal, wherein the desired outcome is increased T cell function, comprising administering to the mammal an effective amount of an agent which inhibits the activity of Rin2.

56. A method of enhancing Ras-dependent cell proliferation in a mammal, comprising administering to the mammal an effective amount of an agent which inhibits the activity of Rin2.

57. A method according to Claim 56, which produces enhanced wound healing, angiogenesis, and/or re-epithelialization.

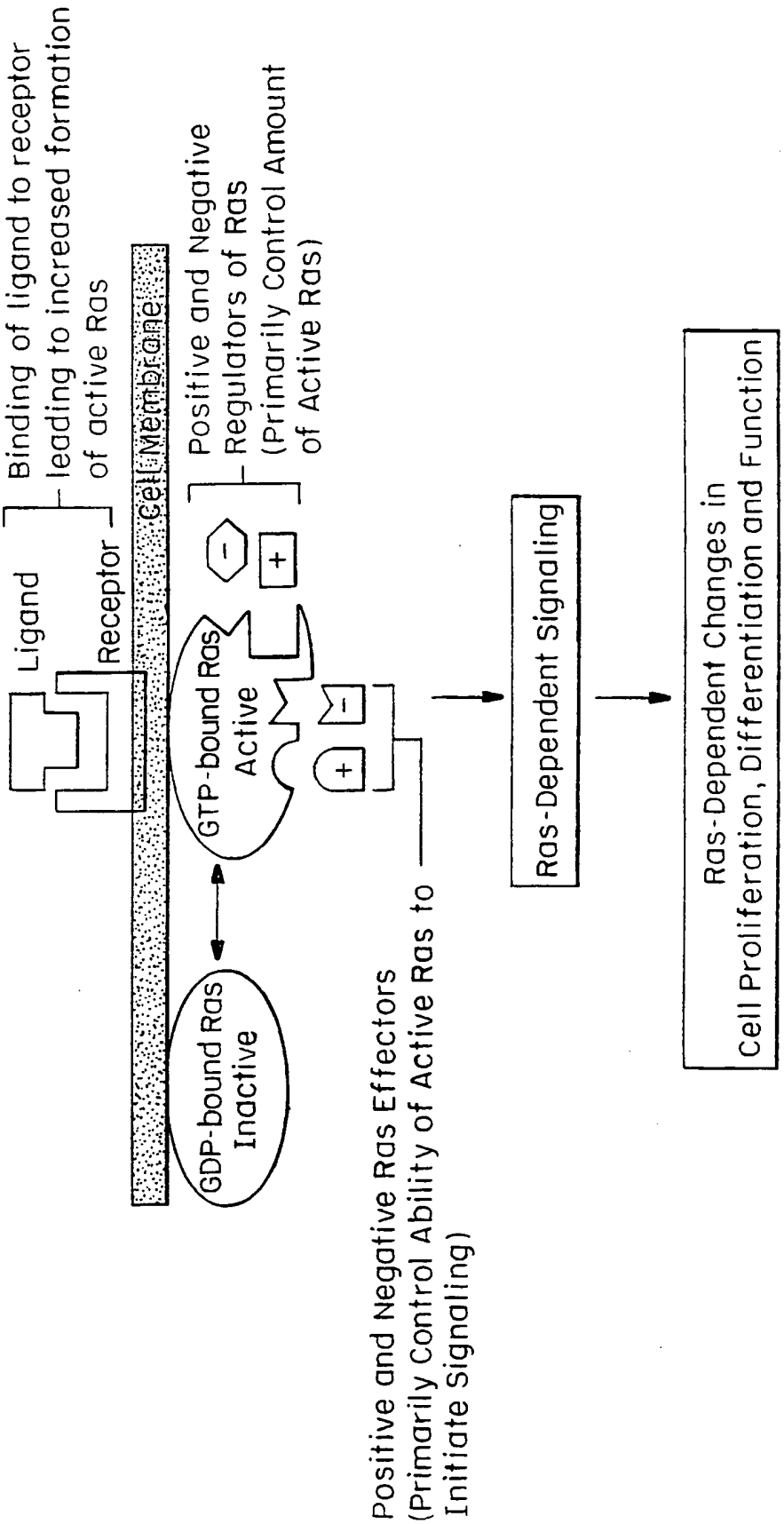
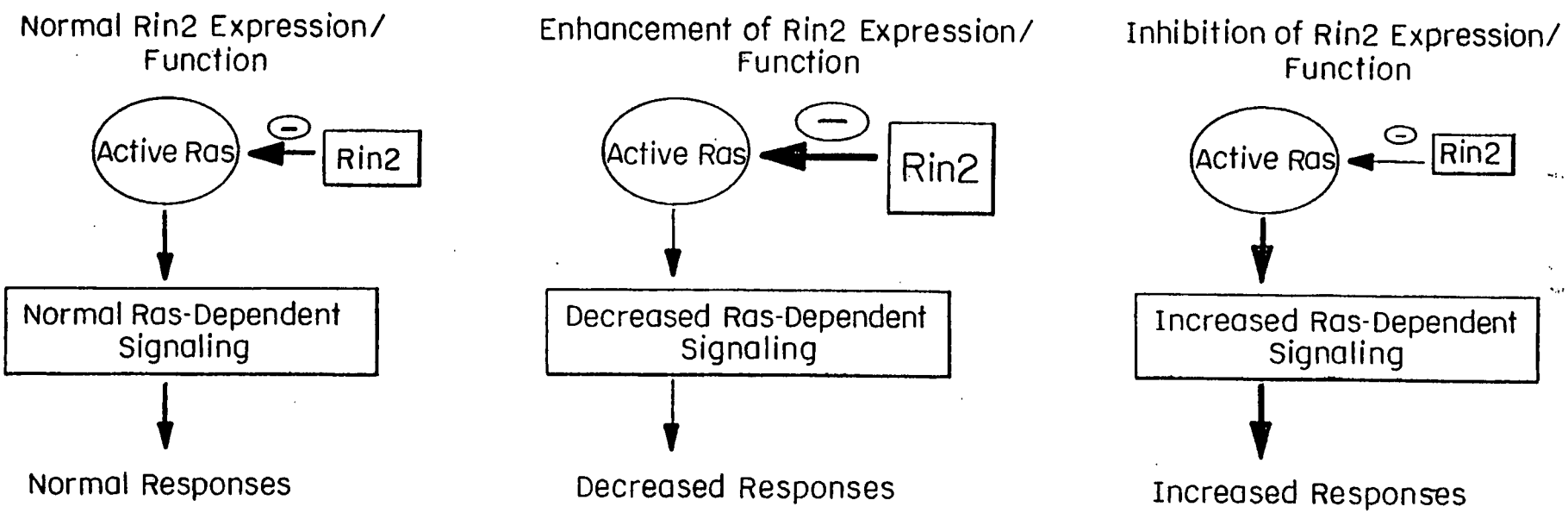

Binding of ligand to receptor leading to increased formation of active Ras
Ligand
Receptor
Cell Membrane
Positive and Negative Regulators of Ras (Primarily Control Amount of Active Ras)
GTP-bound Ras Active
GDP-bound Ras Inactive
Positive and Negative Ras Effectors (Primarily Control Ability of Active Ras to Initiate Signaling)
Ras-Dependent Signaling
Ras-Dependent Changes in Cell Proliferation, Differentiation and Function

FIG. 1



Examples of normal responses regulated by Ras-dependent signaling	Settings in which it is desirable to decrease such responses	Settings in which it is desirable to increase such responses
Mast cell mediator production	Asthma, hay fever, atopic dermatitis	Immunity to pathogens
T cell function	Graft rejection; T cell dependent diseases (certain autoimmune diseases, contact dermatitis, graft-versus-host disease)	HIV infection; certain other infections; immunosupressions associated with cancer therapies
Cell proliferation	Cancer; proliferation of vascular wall cells in arteriosclerosis	Wound healing; angiogenesis (e.g. to improve ischemia; re-epithelialization); nerve regeneration

FIG. 2

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1                                                                    GCACGAGGGGCGCTGGGAGCGGCGGTCGGACGCCGGCGGAGCCGCGGAGCAGGAAGAAG ATG     62
                                                                                                                                    Met     1
63  AGC CTG AAG TCC GAA CGC AGG GGA ATT CAT GTG GAT CAA TCT GAG CTC CTG TGC AAG AAA    122
 2  Ser Leu Lys Ser Glu Arg Arg Gly Ile His Val Asp Gln Ser Glu Leu Leu Cys Lys Lys     21
123 GGA TGC GGT TAC TAC GGC AAC CCT GCC TGG CAG GGT TTC TGC TCC AAG TGC TGG AGG GAG    182
 22  Gly Cys Gly Tyr Tyr Gly Asn Pro Ala Trp Gln Gly Phe Cys Ser Lys Cys Trp Arg Glu     41
183 GAG TAC CAC AAG GCC CGG CAG AGG CAG ATC CAA GAG GAC TGG GAA CTG GCA GAA CGA CTT    242
 42  Glu Tyr His Lys Ala Arg Gln Arg Gln Ile Gln Glu Asp Trp Glu Leu Ala Glu Arg Leu     61
243 CAG CGG GAG GAG GAA GAG GCC TTC GCG AGC AGC CAG AGC AGC CAA GGA GCC CAG TCC CTC    302
 62  Gln Arg Glu Glu Glu Glu Ala Phe Ala Ser Ser Gln Ser Ser Gln Gly Ala Gln Ser Leu     81
303 ACC TTC TCC AAG TTC GAG GAG AAG AAG ACC AAT GAG AAA ACC CGA AAA GTC ACC ACA GTG    362
 82  Thr Phe Ser Lys Phe Glu Glu Lys Lys Thr Asn Glu Lys Thr Arg Lys Val Thr Thr Val    101
363 AAG AAG TTC TTC AGC GCC TCT TCC AGA GCT GGA TCC AAG AAG GAA ATT CAG GAA GCC AAA    422
102  Lys Lys Phe Phe Ser Ala Ser Ser Arg Ala Gly Ser Lys Lys Glu Ile Gln Glu Ala Lys    121
423 GCT CCC AGT CCC TCC ATA AAC CGG CAA ACC AGC ATT GAG ACG GAC CGA GTG ACT AAG GAG    482
122  Ala Pro Ser Pro Ser Ile Asn Arg Gln Thr Ser Ile Glu Thr Asp Arg Val Thr Lys Glu    141
483 TTC ATA GAC TTT CTC AAG ACC TTC CAC AAG ACA GGC CAA GAA GTC TAT AAA CAG ACG AAG    542
142  Phe Ile Asp Phe Leu Lys Thr Phe His Lys Thr Gly Gln Glu Val Tyr Lys Gln Thr Lys    161
543 ATG TTT TTG GAA GCA ATG CCT TAT AAA AGG GAT TTA AGC ATC GAG GAA CAG TCA GAA TGT    602
162  Met Phe Leu Glu Ala Met Pro Tyr Lys Arg Asp Leu Ser Ile Glu Glu Gln Ser Glu Cys    181
603 ACT CAG GAC TTT TAC CAA AAT GTG GCT GAA AGA ATG CAG ACC CGT GGG AAA GTG CCT CCA    662
182  Thr Gln Asp Phe Tyr Gln Asn Val Ala Glu Arg Met Gln Thr Arg Gly Lys Val Pro Pro    201
663 GAG AAA GTG GAG AAG ATA ATG GAT CAG ATC GAA AAG CAC ATC ATG ACG CGT CTC TAT AAA    722
202  Glu Lys Val Glu Lys Ile Met Asp Gln Ile Glu Lys His Ile Met Thr Arg Leu Tyr Lys    221
723 TTT GTG TTC TGC CCA GAG ACT ACT GAT GAT GAG AAG AAA GAT CTC GCC ATT CAA AAA AGA    782
222  Phe Val Phe Cys Pro Glu Thr Thr Asp Asp Glu Lys Lys Asp Leu Ala Ile Gln Lys Arg    241
783 ATC AGG GCC CTG CAC TGG GTA ACG CCT CAG ATG CTC TGT GTC CCT GTC AAT GAG GAA ATC    842
242  Ile Arg Ala Leu His Trp Val Thr Pro Gln Met Leu Cys Val Pro Val Asn Glu Glu Ile    261
843 CCT GAA GTG TCC GAC ATG GTG GTG AAA GCG ATC ACA GAC ATC ATT GAG ATG GAC TCA AAG    902
262  Pro Glu Val Ser Asp Met Val Val Lys Ala Ile Thr Asp Ile Ile Glu Met Asp Ser Lys    281
903 CGT GTG CCT CGG GAC AAG CTG GCC TGC ATC ACC AGG TGC AGC AAG CAC ATC TTC AAT GCC    962
282  Arg Val Pro Arg Asp Lys Leu Ala Cys Ile Thr Arg Cys Ser Lys His Ile Phe Asn Ala    301
963 ATC AAG ATC ACC AAG AAT GAG CCA GCC TCT GCC GAT GAC TTC CTG CCC ACC CTG ATC TAC   1022
302  Ile Lys Ile Thr Lys Asn Glu Pro Ala Ser Ala Asp Asp Phe Leu Pro Thr Leu Ile Tyr    321
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FIG. 3A

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1023  ATC GTC CTG AAG GGC AAC CCC CCT CGC CTG CAG TCC AAC ATC CAG TAC ATC ACT CGC TTC   1082
 322  Ile Val Leu Lys Gly Asn Pro Pro Arg Leu Gln Ser Asn Ile Gln Tyr Ile Thr Arg Phe    341
1083  TGC AAC CCC AGC CGG CTC ATG ACG GGC GAG GAT GGC TAC TAC TTC ACC AAC CTG TGC TGT   1142
 342  Cys Asn Pro Ser Arg Leu Met Thr Gly Glu Asp Gly Tyr Tyr Phe Thr Asn Leu Cys Cys    361
1143  GCT GTG GCT TTC ATT GAG AAA TTA GAC GCC CAG TCT TTG AAT TTA AGT CAG GAG GAT TTT   1202
 362  Ala Val Ala Phe Ile Glu Lys Leu Asp Ala Gln Ser Leu Asn Leu Ser Gln Glu Asp Phe    381
1203  GAC CGG TAC ATG TCT GGC CAG ACA TCC CCC AGG AAG CAG GAG TCT GAG AGT TGG CCC CCG   1262
 382  Asp Arg Tyr Met Ser Gly Gln Thr Ser Pro Arg Lys Gln Glu Ser Glu Ser Trp Pro Pro    401
1263  GAG GCC TGC TTA GGT GTG AAG CAA ATG TAT AAG AAC TTG GAC CTC CTG TCT CAG TTG AAT   1322
 402  Glu Ala Cys Leu Gly Val Lys Gln Met Tyr Lys Asn Leu Asp Leu Leu Ser Gln Leu Asn    421
1323  GAA CGG CAA GAA AGG ATC ATG AAC GAA GCC AAG AAA CTT GAA AAA GAC TTA ATA GAC TGG   1382
 422  Glu Arg Gln Glu Arg Ile Met Asn Glu Ala Lys Lys Leu Glu Lys Asp Leu Ile Asp Trp    441
1383  ACA GAC GGG ATT GCC AAG GAA GTT CAA GAC ATT GTT GAG AAA TAC CCA CTG GAG ATT AAG   1442
 442  Thr Asp Gly Ile Ala Lys Glu Val Gln Asp Ile Val Glu Lys Tyr Pro Leu Glu Ile Lys    461
1443  CCC CCG AAC CAA CCC TTA GCA GCC ATC GAC TCT GAG AAT GTG GAG AAC GAC AAG CTC CCT   1502
 462  Pro Pro Asn Gln Pro Leu Ala Ala Ile Asp Ser Glu Asn Val Glu Asn Asp Lys Leu Pro    481
1503  CCC CCT CTG CAG CCT CAG GTG TAC GCA GGG TGACGGCCCTGTTTATTTGGGGCTGGTTTCTGGGAGCTG   1571
 482  Pro Pro Leu Gln Pro Gln Val Tyr Ala Gly ***                                       491

1572  CTGCGTTCCACTGTTCAGGTCCGGAATATGAACTGACTGCTTAAAGTTTCAAAGTGTTTTTAGGTACAGATTTAGGGAT   1650
1651  TGGTTATTCTCTTTTTTCTTCTCTAGCGGGGAAGCTTAGTAAATAATAATGTACTATTTATTTGAGCTGGTGGAGTAGG   1729
1730  TTTGTGTGAATTCTGTGTCGCTCTTTTATGTCCTGCCTGATTTCCCATGGGCTTCCTCTGTGTAGACACTGTTGTTGGT   1808
1809  TTTGGGCAAACACTGCCTTTTAAAGGATAAAACAGATGCTATAAAGTCTATGTTGAAATGAATTCTATGTTCCCACACT   1887
1888  CCCCCAGTGTGAAATAATTTTGTAATTGTAAAGATAGAAGATAATGTTAATAAGTAAATATGTAAAATTGTAAATATGT   1966
2967  AAAAAAAAAACACATAGGGCTGGGGAGGGGTGTCTCAGCGTGCATGGCATTTCATGAGCTGATGTTTTTTTTTTTTTT   2045
2046  TAGGTGAAAATGAAATTTATTGAATGTTTGCCTTTAGCGCCATTTTATATGGTTTGTCCCACTAAAAAGAATCTAAAGA   2124
2125  ATTTGAGCTTTAACAGGACATTGGCACTAACTGCCCTAACTTGAGATTCTTTCTGGTACATGTGAAGAAGTTGTAACGC   2203
2204  CAACTTTTAGGTCACATACAGAATTATTCTGGGACCCTGGGGTGGTGGCTCAGTCAGTAAAGTGCTCTCCTATACTGAT   2282
2283  GTGAGGACCTGAGCTCCCTGTCCGGCCCCGGGTGAAAAGCTGGGCATGGTGACACTCACTTGGGACAGCTCTGCTGGGA   2361
2362  GCCAGAGTTCCTGGGGCAGGGGGCATCGCTGTCAATGAGACACCTCGTGTAAGCAAACAAATCAAGATGGACGGCTCCT   2440
2441  GAGAAATGATAGCCAAGGATGCCCTCTGGCCTCCACATGGCCACATATGTGCCTGTGTACCTCTACATACATGTGTAGC   2519
2520  ACACACACATGAACACACAGTTGCTGATTAGTACAGTTGACTTGGAACTGTGCTTGCAGCTTCCTTTCCCTGTTTATCC   2598
2599  AATAAACTTCCCCCACAGTGCTGTGGGGCTATTGCCTTTTTTATCTGAAAAAAAAAAAAAAAAAAA                2664
```

FIG. 3B

```
Rin2     227   ETTDDEKKDL AIQKRIRALH WVTPQMLCVP VNEEIPEVSD MVVKAITDII
Vps9p    157   EHMKDLTNDD TLLEKIRHYR FISPIMLDIP DTMPNARLNK FVHLASKELG
Rin1     155   RLAADG-SLG RLAEGLRLAR AQGPGAFGSH LSLPSPVE-- -LEQVRQKLL
JC265    182   FHMADG-SWK QLKENLQLVR QRNPQELGVF APTPDFVD-- -VEKIKVKFM

Rin2     277   EMDSKRVPRD KLACITRCSK HIFNAIKITK NEPASADDFL PTLIYIVLKG
Vps9p    207   KINRFKSPRD KMVCVLNASK VIFGLLKHTK LEQNGADSFI PVLIYCILKG
Rin1     201   QLVRTYSPSA QVKRLLQACK LLYMALRTQE GEGSGADGFL PLLSLVLAHC
JC265    228   TMQKMYSPEK KVMLLLRVCK LIYTVMENNS GRMYGADDFL PVLTYVIAQC

                         [LL  CK LIY]        [GAD FL PVL]
                          MOTIF I             MOTIF II

Rin2     327   NPPRLQSNIQ YITRFCNPSR LMTGEDGYYF TNLCCAVAFI EKLDAQSL
Vps9p    257   QVRYLVSNVN YIERFRSPDF I-RGEEEYYL SSLQAALNFI MSLTERSL
Rin1     251   DLPELLLEAE YMSELLEPSL L-TGEGGYYL TSLSASLALL SGLGQAHT
JC265    278   DMLELDTEIE YMMELLDPSL L-HGEGGYYL TSAYGALSLI KNFQEEQA

                              [GE  YYL TS]
                               MOTIF III
```

FIG. 4

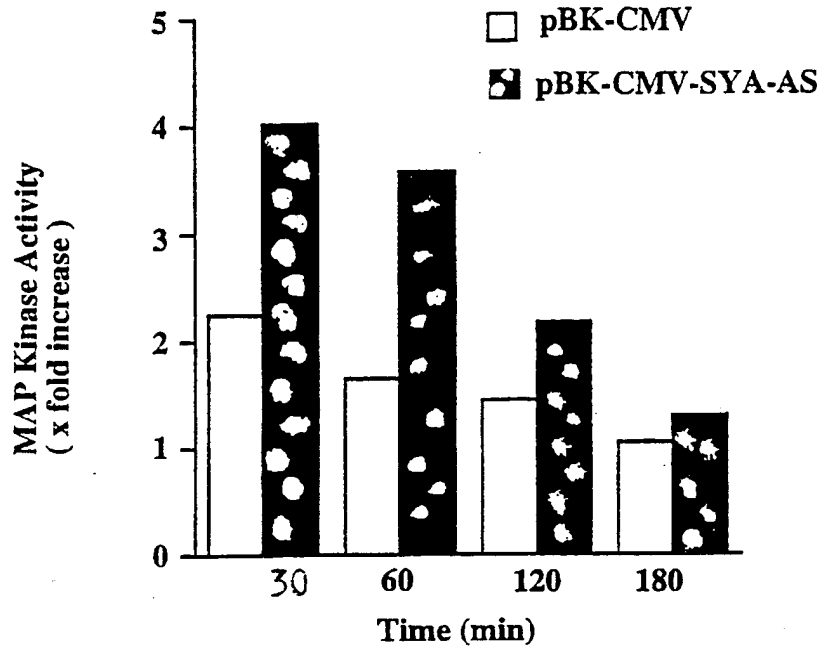
pBK-CMV
pBK-CMV-SYA-AS
MAP Kinase Activity
(x fold increase)
0
1
2
3
4
5
30
60
120
180
Time (min)

FIG. 5

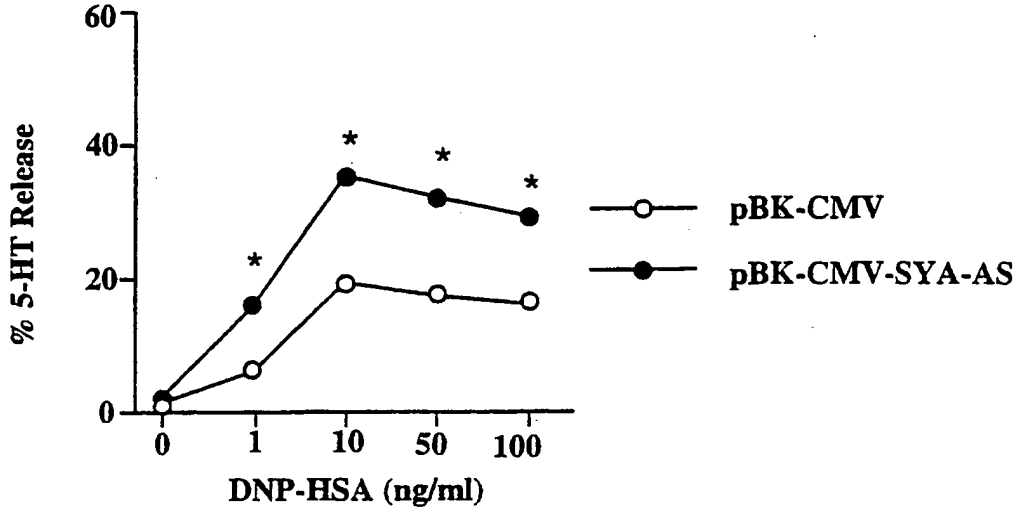
% 5-HT Release
0
20
40
60
0
1
10
50
100
DNP-HSA (ng/ml)
*
*
*
*
pBK-CMV
pBK-CMV-SYA-AS

FIG. 6

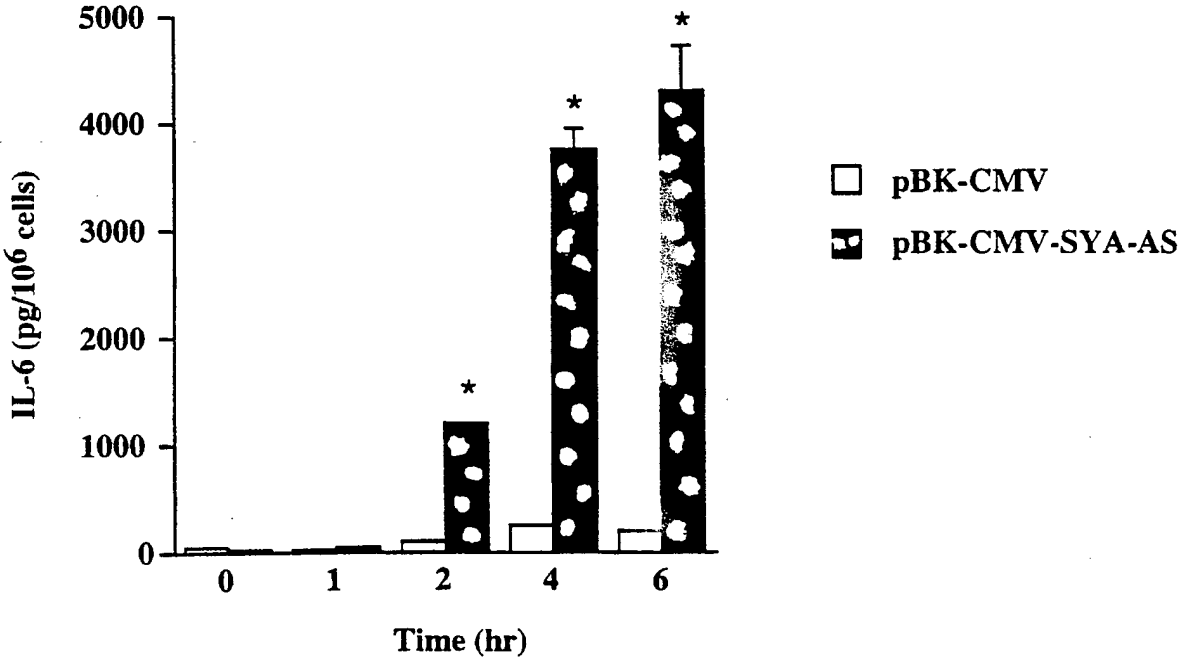
5000
4000
3000
2000
1000
0
IL-6 (pg/10^6 cells)
0
1
2
4
6
Time (hr)
*
pBK-CMV
pBK-CMV-SYA-AS

FIG. 7A

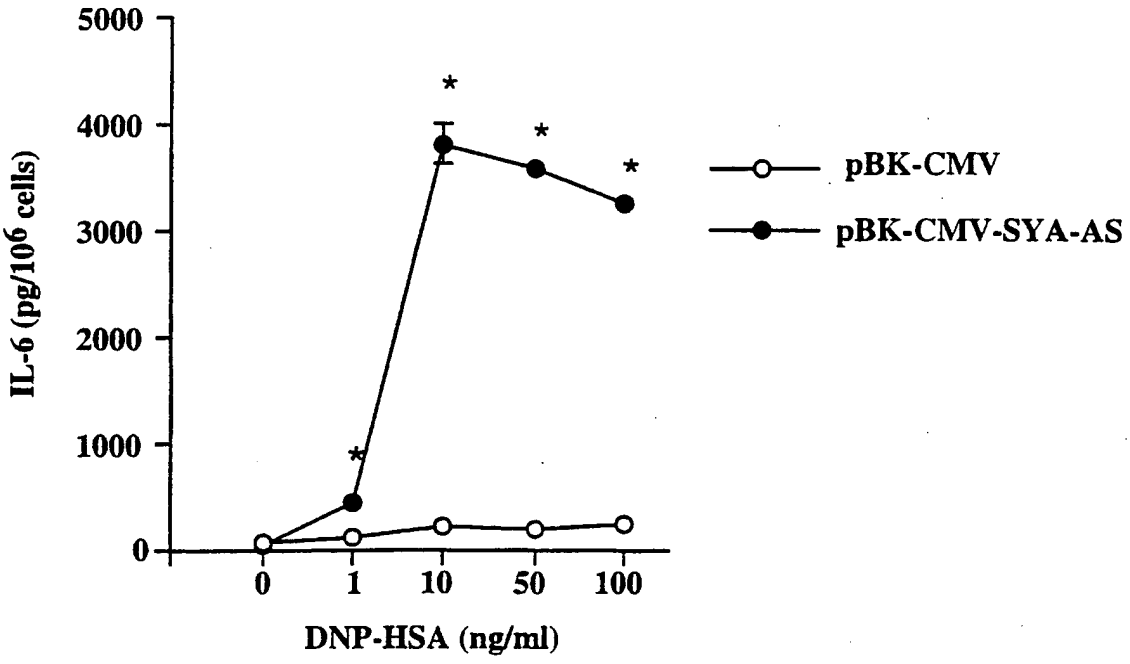
5000
4000
3000
2000
1000
0
IL-6 (pg/10^6 cells)
0
1
10
50
100
DNP-HSA (ng/ml)
*
pBK-CMV
pBK-CMV-SYA-AS

FIG. 7B

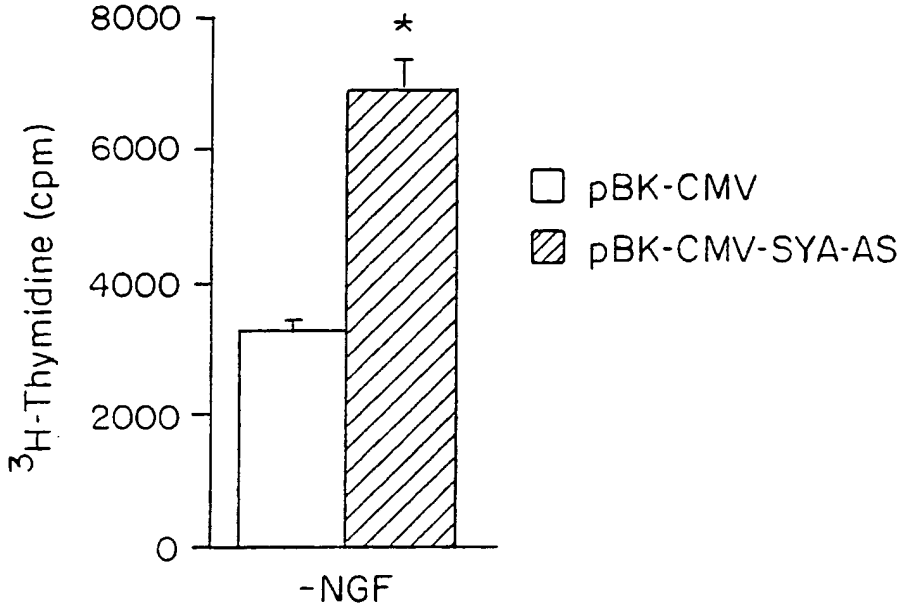
8000
6000
4000
2000
0
*
^{3}H-Thymidine (cpm)
-NGF
pBK-CMV
pBK-CMV-SYA-AS

FIG. 8A

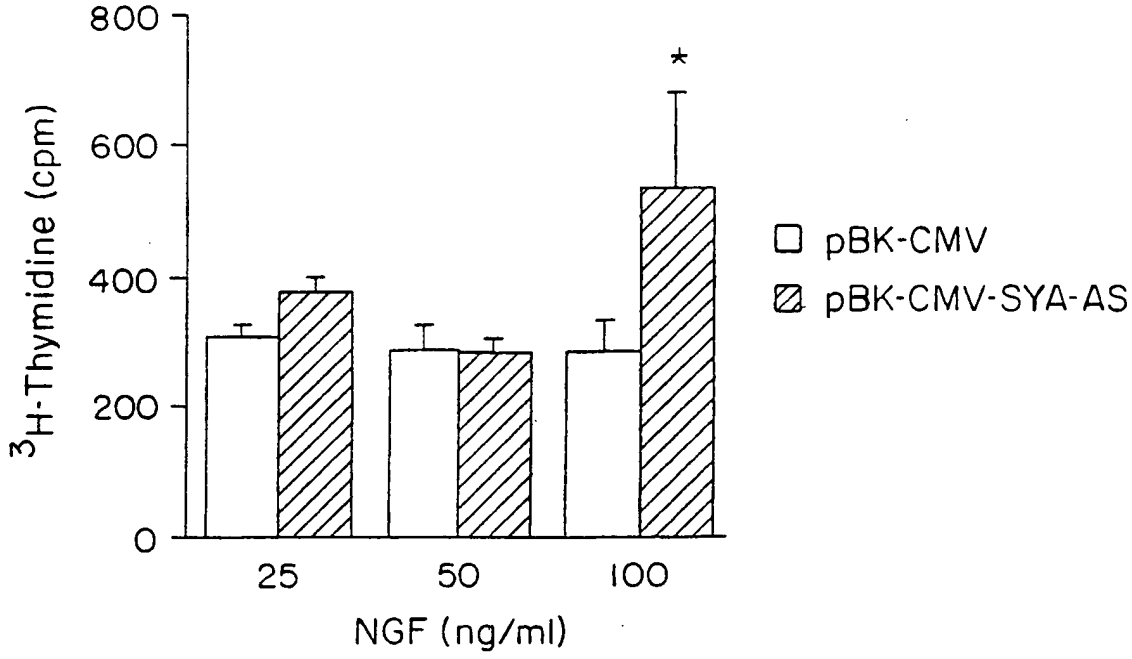
800
600
400
200
0
*
^{3}H-Thymidine (cpm)
25
50
100
NGF (ng/ml)
pBK-CMV
pBK-CMV-SYA-AS

FIG. 8B

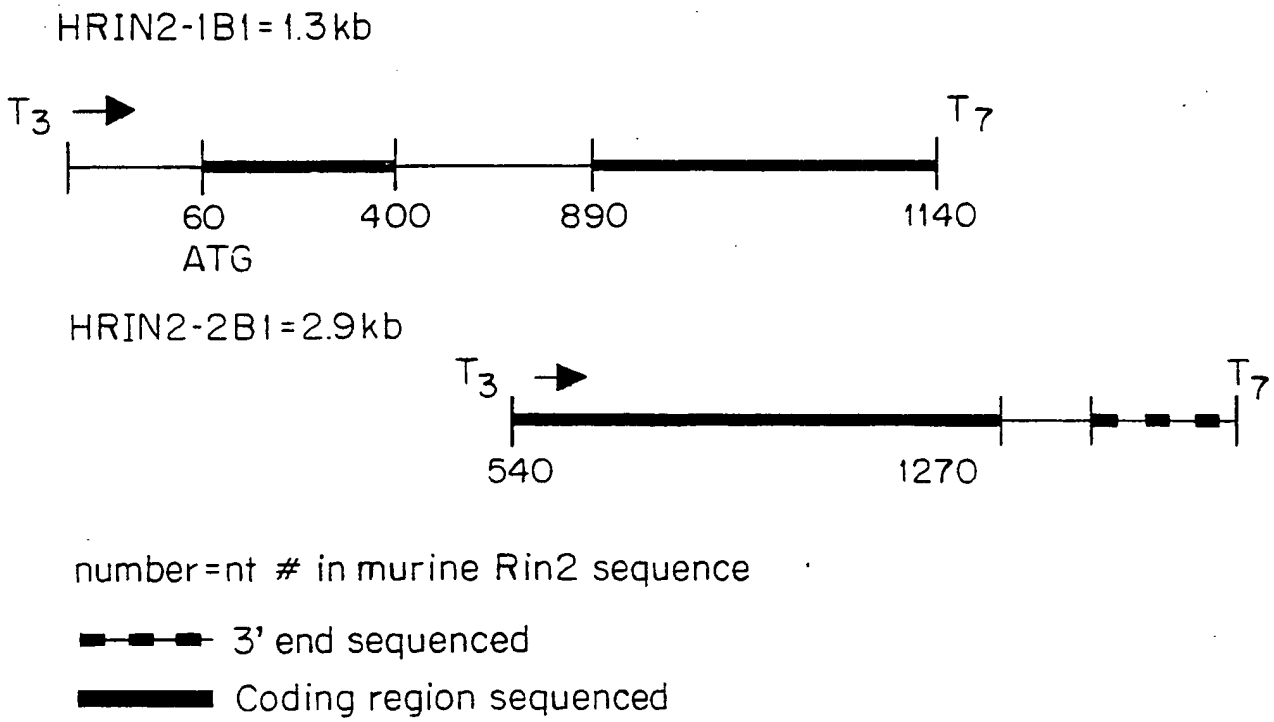
HRIN2-1B1=1.3kb
T3
T7
60
ATG
400
890
1140
HRIN2-2B1=2.9kb
T3
T7
540
1270
number=nt # in murine Rin2 sequence
3' end sequenced
Coding region sequenced

FIG. 9

79.2% identity in 355 nt overlap; score: 883

```
Murine Rin2
                60        70        80        90       100       110
_       AGGAAGAAGATGAGCCTGAAGTCCGAACGCAGGGGAATTCATGTGGATCAATCTGAGCTC
        : ::::::::::::::: : ::: :::::: : ::::::::::::::::
_       AAGAAGAAGATGAGCCTTAGGTCTGAACGCCGAGGAATTCATGTGGATC----------

HRIN2-1B1
               120       130       140       150       160       170
_       CTGTGCAAGAAAGGATGCGGTTACTACGGCAACCCTGCCTGGCAGGGTTTCTGCTCCAAG
        :::::::::::::::: :::::::  ::::::::: :::::::::::::::::::::::
_       CTGTGCAAGAAAGGATGTGGTTACTGTGGCAACCCTACCTGGCAGGGTTTCTGCTCCAAG

               180       190       200       210       220       230
_       TGCTGGAGGGAGGAGTACCACAAGGCCCGGCAGAGGCAGATCCAAGAGGACTGGGAACTG
        :::::::::::: ::::: ::::: ::: :::::: :::::: :: ::: ::::::: :::
_       TGCTGGAGGGAAGAGTAGCACAAAGCCAGGCAGAAGCAGATTCAGGAGTACTGGGAGCTG

               240       250       260       270       280       290
_       GCAGAACGACTTCAGCGGGAGGAGGAAGAGGCCTTCGCGAGCAGCCAGAGCAGCCAAGGA
        :  :::::::: :..::::::::: :::::: :::: :: :.::: ::::::::::::::
_       GTGGAACGACTCCNNCGGGAGGAAGAAGAG-CCTTTGCCANCAGTCAGAGCAGCCAAGGG

               300       310       320       330       340       350
_       GCCCAGTCCCTCACCTTCTCCAAGTTCGAGGAGAAGAAGACCAATGAGAAAACCCGAAAA
        ::::: :::::::  ::::::::. :: :: :  ::::: ::::: .::::. ::::. ..
_       GCCCAATCCCTCATATTCTCCANCTTTGAAGGAAAGAAAACCAACNAGANCACCCNCNNG

               360       370       380       390       400
_       GTCACCACAGTGAAGAAGTTCTTCAGCGCCTCTTCCAGAGCTGGATCCAAGAAGG
        :: ::::::::::::.:: : ::::::  : :::::::: :  ::::: :: :: :
_       GTTACCACAGTGAANAAAT-CTTCAGTACGTCTTCCAGGGTCGGATCAAAAAAAG
```

FIG. 10A

78.6% identity in 266 nt overlap; score: 607

```
Murine Rin2
                890         900         910         920         930         940
_       ATTGAGATGGACT--CAAAGCGTGTGCCTCGGGACAAGCTGGCC-TGCATCACC-AGGTG
        ::::: ::::: :  ::  : :::::::::::: :: :::::::::: :::.:::::: : : :
        ATTGAAATGGATTNCCAGGGGGTGTGCNTCGAGAAAAGCTGGCCNTGCNTCACCCAAGGG
HRIN2-1B1

                950         960         970         980         990        1000
_       CAGCAAGCACATCTTCAATGCCATCAAGATCACCAAGAATGAGCCAGCCTCTGCCGATGA
        ::::::::.:.:: ::. :.:::::::::::::::  :::: ::::  :: :: :: :::::
_       CAGCAAGNANATTTTNGANGCCATCAAGATCACTTAGAACGAGCTGGCGTCAGCAGATGA

                1010        1020        1030        1040        1050
_       CTTCCTGCCC-ACCCTGATCTACATCGTCCTGAAGGGCAACCCCCCCTCGCCTGCAGTCCA
        .:: :: ::: ::::: :: ::::: ::  ::::::::::::::: : :::: ::::  :
_       NTTTCTTCCCCACCCTCATTTACATTGTTTTGAAGGGCAACCCCCAT-GCCTTCAGTTTA

        1060        1070        1080        1090        1100        1110
_       ACATCCAGTACATCACTCGCTTCTGCAACCCCAGCCGGCTCATGACGGGCGAGGATGGCT
        : :::::::: ::::: :::::::::::: :: ::::: :: ::::: :: ::::::::::
_       ATATCCAGTATATCACGCGCTTCTGCAATCCAAGCCGACTGATGACTGGAGAGGATGGCT

        1120        1130        1140
_       ACTACTTCACCAACCTGTG-CTGTGC
        :::: :::::::: ::: : ::: ::
_       ACTATTTCACCAATCTGAGGCTGGGC
```

FIG. 10B